

***Coupled and Uncoupled Operational Mode
of Nitric Oxide Synthase and the Regulation
of the Sarcolemmal Na⁺ - K⁺ ATPase:
Receptor and Non-receptor Pathways***

Dr. Caroline White MRCPI

Supervisor:

Prof. K. Daly,

National University of Ireland, Galway

May 2012

Acknowledgments

My sincere thanks to Professor Helger Rasmussen who gave me the opportunity to do this research and who was a constant source of encouragement throughout.

To Elisha Hamilton and Alvaro Garcia who were the most patient teachers and colleagues. Without them none of this would have been possible.

To Gemma Figtree who was an immense support throughout.

To my family who have supported me always especially my Nana White who gave me inspiration to see the world in a slightly different way.

To David for everything.

Summary of contents

The membrane Na⁺-K⁺ pump transports 3 Na⁺ ions out and 2 K⁺ ions into cells against their electrochemical gradient, using energy derived from hydrolysis of ATP. The Na⁺ and K⁺ ion gradients generated by the pump serve in secondary co- and counter transport processes critical for cell function. This thesis examined the hypothesis that synthesis of radical oxygen species/radical nitrogen species (ROS/RNS), coupled to hormone receptors and their intracellular messenger pathways, can regulate Na⁺-K⁺ pump activity in cardiac myocytes.

A whole cell patch clamp technique was used measure the membrane current generated by the Na⁺-K⁺ pump (I_p) of isolated rabbit cardiac myocytes.

Supplementing the patch pipette solution with L-arginine (L-Arg), a known substrate of nitric oxide synthase(NOS) stimulated Na⁺-K⁺ pump activity in a manner sensitive to the soluble guanylate cyclase inhibitor 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ). In contrast, uncoupling NOS by exposing myocytes to the chemical paraquat, caused significant Na⁺-K⁺ pump inhibition which was abolished by ROS/RNS scavengers, ebselen, and superoxide dismutase(SOD).

Since Angiotensin II (AngII) activates nicotinamide adenine dinucleotide phosphate hydrogen (NAD(P)H) oxidase, we tested the hypothesis that NAD(P)H oxidase mediates Ang II-induced pump inhibition. Ang II significantly inhibited the Na⁺-K⁺ pump. This was abolished by the addition of apocynin and gp91ds potent NAD(P)H oxidase inhibitors. The effect was also abolished by the addition of ε protein kinase C (εPKC) inhibitor. Forskolin, an activator of adenylyl cyclase was used to mimic β₁/β₂ adrenergic receptor activation. It

decreased electrogenic $\text{Na}^+\text{-K}^+$ pump current (I_p). Interestingly, in addition to the expected role of protein kinase A (PKA) in this inhibition, inclusion of specific inhibitors implicated PKC and NADPH oxidase, i.e. cross talk between PKA and the same oxidative signalling pathway we had observed for Ang II.

In summary, we have shown that PKC and PKA activate NADPH oxidase dependent inhibition of the $\text{Na}^+\text{-K}^+$ pump. The data provides a link between ROS/RNS and signalling mediated by neurohormonal activation in the control of cardiac myocyte Na^+ , and by inference, Ca^{2+} and may have important therapeutic implications in heart failure. This work has led to the very recent identification by our laboratory of the specific molecular mechanism by which oxidant signalling inhibits the $\text{Na}^+\text{-K}^+$ pump.

Table of Contents

ABBREVIATIONS	10
Chapter 1	15
General Introduction and Aims.....	15
1.1 The cell and the membrane Na ⁺ -K ⁺ pump.	15
Figure 1.1 Cardiac action potential.....	17
1.2. Key features of the Na ⁺ -K ⁺ pump	22
1.2.1. Structure of Na ⁺ -K ⁺ pump.....	22
Figure1.2 The crystalline structure of the Na ⁺ -K ⁺ ATPase.....	25
1.2.2. The Na ⁺ -K ⁺ pump cycle.....	25
Figure 1.3 The Na ⁺ -K ⁺ pump cycle as described by the Albers-Post reaction scheme	27
1.3. Regulation of the Na ⁺ -K ⁺ pump.....	28
1.3.1. Regulation of the Na ⁺ -K ⁺ pump by its ligands, Na ⁺ , K ⁺ and ATP.....	28
1.3.2. Voltage dependence of the Na ⁺ -K ⁺ pump	29
Figure 1.4 Access channel model	30
1.4. Na ⁺ -K ⁺ pump regulation: Phosphorylation or Reactive Oxygen Species?..	31
1.4.1. Role of phosphorylation in Na ⁺ -K ⁺ pump regulation.....	31
1.4.2. Potential role of reactive oxygen and nitrogen species.....	32
Figure 1.5 Sources of ROS in vascular cells	35
1.4.3. NADPH oxidase.....	36
1.4.4. Nitric oxide synthase.....	39

Figure 1.7 Structure and interactions of Nitric Oxide Synthase	41
Figure 1.8 Schematic representation of Nitric Oxide Synthase (NOS)	
uncoupling	44
1.4.5. Metabolic Pathways for O ₂ ⁻	45
1.4.6. Superoxide Dismutase.....	47
1.5. The Renin-Angiotensin system, adrenergic signaling and the Na ⁺ -K ⁺ pump: A role for ROS/RNS in signalling?	48
1.5.1. Angiotensin II.....	50
Figure 1.10 Angiotensin II and its receptor activation	53
1.5.2. β-Adrenergic signalling.	54
1.6. Scope of thesis.	55
Chapter 2.....	57
Methods.....	57
2.1. Animals and housing.....	57
2.2. Isolation of Cardiac Myocytes.	57
2.3. The Whole Cell Patch Clamping Technique.....	60
Figure 2.1 The whole-cell patch-clamp technique	61
2.3.1 Tissue bath and experimental set up	62
2.3.2. Patch clamp equipment.....	63
2.3.3. Experimental Solutions.	64
2.3.4. Pipette filling solutions.....	65
2.3.5. Measurement of membrane capacitance and access resistance.....	66

2.3.6. Measurement of I_p	67
Figure 2.2 Trace of ouabain induced shift in holding current.....	68
2.4. Data acquisition, analysis and storage.....	69
Chapter 3.....	71
Regulation of the cardiac Na-K pump by NOS- derived nitric oxide.....	71
3.1. Introduction.	71
3.2. Methods.	72
3.3. Results.....	74
Figure 3.1 Effect of L-arginine on Na^+K^+ pump current in myocytes	75
Figure 3.2 Effect of L-arginine on I_p with 0 External Na^+	77
Figure 3.5 The effect of ODQ on L-arg induced Na^+K^+ pump stimulation	80
Figure 3.6 Effect of BH_4 on control myocytes and effect of BH_4 on L-arg induced Na^+K^+ pump stimulation	82
3.4. Discussion.....	83
Chapter 4.....	86
Uncoupling of Nitric Oxide Synthase.....	86
4.1 Paraquat induced uncoupling of NOS.....	86
Figure 4.1 Electron flow in the reductase domain of NOS I in the presence of paraquat	87
4.2. Methods	88
4.3. Results.....	88
Figure 4.2 Effect of paraquat on L-arginine induced Na^+K^+ pump stimulation...	90

Figure 4.4 Effect of superoxide scavenger Ebselen on paraquat induced Na ⁺ -K ⁺ pump inhibition	93
4.4.Discussion.....	94
Chapter 5	96
5.1. NADPH oxidase as an endogenous source of O ₂ ⁻ in Angiotensin II induced Na ⁺ -K ⁺ pump inhibition.....	96
5.2. Experimental solutions.....	98
5.3. Results.....	99
Figure 5.2 Role of superoxide and NADPH oxidase in Ang II-induced Na ⁺ -K ⁺ pump inhibition.....	103
Figure 5.3 Role of PKC in Ang II-induced Na ⁺ -K ⁺ pump inhibition.....	105
Figure 5.4 Oxidant stress and PKC-dependent Na ⁺ -K ⁺ pump inhibition	107
5.4. Discussion.....	108
5.5.1. Functional studies.....	108
Figure 5.5 Co-immunoprecipitation of the Na ⁺ -K ⁺ pump with caveolin and NADPH oxidase subunits.....	110
5.5.2. Ang II induces a decrease in pump affinity for Na ⁺	112
Chapter 6	120
NADPH oxidase and β-adrenergic regulation of the Na ⁺ -K ⁺ pump.	120
6.1 Introduction	120
6.2. Methods and experimental solutions.....	122
6.3. Results.....	124

Figure 6.1 Effect of forskolin on Na ⁺ -K ⁺ pump current in myocytes	127
Figure 6.2. Effect of forskolin on near-maximal I _p	129
Figure 6.3 Role of superoxide and NADPH oxidase in forskolin-induced Na ⁺ -K ⁺ pump inhibition.....	132
Figure 6.4 Role of PKA and PKC in forskolin-induced Na ⁺ -K ⁺ pump inhibition... ..	134
6.4. Discussion.....	135
Figure 6.5 Effects of inhibitors of PKA, εPKC and PLC on forskolin-induced Na ⁺ -K ⁺ pump inhibition.....	140
Figure 6.6 Effect of forskolin on glutathionylation of the Na ⁺ -K ⁺ pump's β ₁ subunit	143
Figure 6.7 Summary of molecular- fluorescence- and patch clamp studies on effects of forskolin	144
Chapter 7 Discussion.....	145
7.0. Implications and Perspective.	145
7.2. Nitric oxide and the Na ⁺ -K ⁺ pump.	146
7.3. ROS/RNS and the Na ⁺ -K ⁺ pump.....	148
7.4. Na ⁺ -K ⁺ pump and Heart Failure.	158
7.4.3 Role of ROS /RNS in Heart Failure.....	160
7.4.4 Energy Starvation	162
7.5 Unifying Scheme and implications for future work	163
8.0 Bibliography	166

ABBREVIATIONS

AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ANG II	Angiotensin II
ANP	Atrial natriuretic peptide
AR	Adrenergic receptor
ARB	Angiotensin II <i>receptor blockers</i>
ATP	Adenosine triphosphate
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cANP(4-23)	NPR-C specific atrial natriuretic peptide (amino acids 4 – 23)
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
C _m	Membrane capacitance
CNG	Cyclic nucleotide-gated
CNP	C-type natriuretic peptide
DAF	Diaminofluorescein
DAG	Diacylglycerol
DHE	Dihydroethidium
DTPA	<i>Diethylene triamine pentaacetic acid</i>
DTT	Dithiothreitol
EGTA	Ethyleneglycol-bis-(β -aminoethylether) <i>N,N,N',N'</i> -tetraacetic acid
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
Epac	Exchange protein directly activated by cAMP
GC	Guanylyl cyclase
GEE	Glutathione ethyl ester
G _i	Inhibitory guanine nucleotide regulatory protein
GRx1	<i>Glutaredoxin-1</i>
G _s	Stimulatory guanine nucleotide regulatory protein
GSH	Glutathione

GSS ⁻	Glutathionylation
GSSG	Glutathione disulfide
H ₂ O ₂	Hydrogen peroxide
H-89	N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrate dihydrochloride
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HF	Heart failure
IB	Immunoblot
IC ₅₀	Concentration giving half maximal inhibition
IgG	Immunoglobulin G
I _p	Pump current
IP	Immunoprecipitate
K ⁺ _i	Intracellular K ⁺
KHB	Krebs-Henseleit buffer
K _m	Concentration of substrate that leads to half-maximal velocity
L-NAME	NG-nitro-L-arginine methyl ester
Na ⁺ _i	Intracellular Na ⁺
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEM	<i>N</i> -ethylmaleimide
NMG-Cl	N-methyl-D-glucamine chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	Non-phagocytic NADPH oxidase
NP	Natriuretic peptide

O ₂ ⁻	Superoxide
ODQ	1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one
OH ⁻	Hydroxide
ONOO ⁻	Peroxynitrite
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PEGylated	Polyethylene glycosylated
pGC	Particulate guanylyl cyclase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Cyclic GMP-dependent protein kinase or protein kinase G
PLC	Phospholipase C
PLM	Phospholemman
PLMS	Phospholemman -like protein from shark rectal gland
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
R _a	Access resistance
RACK	Receptor for activated C kinase
RICK	Receptor for inhibited C kinase
RIPA	Radioimmunoprecipitation assay buffer
RNS	Reactive nitrogen species
RNSH	Royal North Shore Hospital
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SNP	Sodium nitroprusside
SNAP	S-nitroso-L-acetyl penicillamine
SOD	Superoxide dismutase
T-1032	methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline

	carboxylate sulfate
TEA-Cl	Tetraethylammonium chloride
TMA-Cl	Tetramethylammonium chloride
U73122	1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione
V_m	Membrane voltage
V_{max}	Maximum velocity of enzyme
XO	Xanthine oxidase
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

Chapter 1

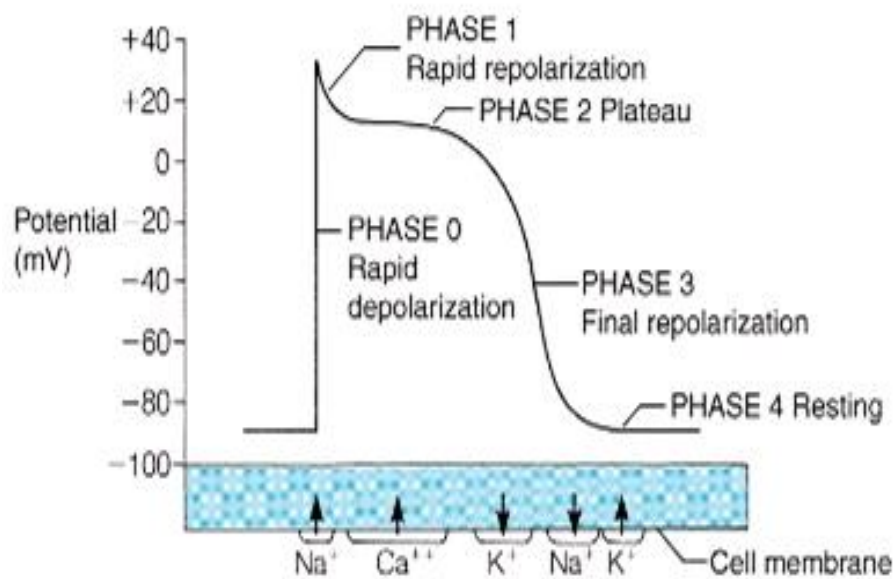
General Introduction and Aims.

1.1 The cell and the membrane Na⁺-K⁺ pump.

All eukaryotic cells are characterised by steep trans-membrane electrochemical gradients for sodium (Na⁺) and potassium (K⁺) with a high concentration of Na⁺ on the outside and a low concentration on the inside. The converse applies for outside and inside K⁺ concentrations. These trans-membrane gradients are maintained by the energy-dependent “uphill” transport of Na⁺ out of and K⁺ into cells, against their respective electrochemical gradients. The transport is mediated by Na⁺-K⁺ pump molecules embedded in the cell membrane. This pump transports three Na⁺ ions out of the cell in exchange for two K⁺ ions transported in. The energy for this process is derived from the hydrolysis of one ATP molecule in each pump cycle (Taub et al., 2004, Therien and Blostein, 2000). Because of this dependence on energy derived from ATP, the process is regarded as “active transport”. In most cell types, the pump consumes more energy than any other single process. It is responsible for at least 15-20% of the resting energy expenditure in an organism (Clausen et al., 1991).

The Na⁺ and K⁺ gradients play a particularly important role in the excitation of “excitable cells”, *i.e.* cells capable of generating an action potential. The trans-membrane electrochemical gradients for Na⁺ and K⁺ are key determinants of the cells’ membrane potentials, predominantly determined by the ion with the

highest membrane permeability. In the resting state of cells, K^+ typically has the highest permeability. The membrane potential of the cell is shifted towards the potential at which the chemical driving force arising from the concentration gradient across the membrane is exactly counterbalanced by the electrical driving force acting on the K^+ ions. This potential is known as the equilibrium potential for K^+ (E_K). When an action potential is triggered, the membrane permeability for Na^+ rapidly increases and the Na^+ gradient becomes the predominant determinant of the membrane potential which transiently shifts towards the equilibrium potential for Na^+ (E_{Na}). The Na^+ permeability subsequently inactivates, and the membrane potential returns towards E_K . Figure 1.1 illustrates some of the key features that determine the cardiac action potential.



Adapted from Lippincott Williams & Wilkins 2004

Figure 1.1 Cardiac action potential

Phase 0 (depolarisation) is primarily due to a rapid increase in Na^+ conductance accompanied by a fall in K^+ conductance. **Phase 1:** repolarisation represents a rapid shift in K^+ movement. K^+ channels are briefly opened causing a transitory, hyperpolarizing outward K^+ current. Simultaneously, there is an increase in the amount of slow inward Ca^{2+} current (long-lasting L-type calcium channels) and given the brief nature of outward K^+ current, repolarisation is delayed, This then give the plateau phase in the action potential known as **Phase 2** (characteristic of cardiac action potentials). **Phase 3:** repolarisation occurs when outward K^+ ion movement increases, along with the inactivation of Ca^{2+} channels. Therefore, the action potential in non-pacemaker myocytes is principally determined by relative changes in fast Na^+ , slow Ca^{2+} and K^+ gradients and transmembrane flux (see text for further discussion). The arrows indicate the time and direction of movement of ions that influence the membrane potential.

In addition to emphasising the role of ion channel currents carried by Na^+ and K^+ , Figure 1.1 also illustrates the contribution Ca^{2+} makes to the action potential. However, this contribution ultimately depends on the activity of the Na^+ - K^+ pump because the trans-membrane gradients maintained by the pump serve in co- and counter-transport processes that determine the intracellular concentration of other ions or mediate cellular uptake of organic compounds. It is of particular relevance to the cardiac myocyte that trans-membrane transport of Ca^{2+} ions is coupled to exchange for Na^+ moving in the opposite direction in a process known as Na^+ - Ca^{2+} exchange. This exchange is voltage-dependent and under most conditions, at negative membrane potentials, it mediates the outward transport of Ca^{2+} in exchange for the inward transport of Na^+ . However, the process can reverse direction at positive potentials and mediate cellular Ca^{2+} uptake. Transport of Ca^{2+} mediated by Na^+ - Ca^{2+} exchange is regarded as “secondary active transport” because it derives its energy from the trans-membrane Na^+ gradient maintained by the ATP-dependent Na^+ - K^+ pump. In most mammals, including man, the main mechanisms of controlling intracellular Ca^{2+} are, calcium induced calcium release (CICR) and SERCA 2a (sarcoendoplasmic reticulum (SR) calcium transport ATPase) mediated Ca^{2+} uptake into the SR. The Na^+ - Ca^{2+} exchanger is also however an important determinant of cytosolic Ca^{2+} in cardiac myocytes and it plays a pivotal role in the coupling of the action potential to myocyte contraction, a process usually referred to as “excitation-contraction coupling” (Bers et al., 2003, Bers, 2001). Cellular concentrations of monovalent ions are also dependent on secondary active transport *via* co- and counter-transporters that derive their energy from the trans-membrane gradient for Na^+ . Examples include the Na^+ - K^+ - 2Cl^- co-

transporter (NKCC). This co-transporter mediates the coupled transport of a Na^+ and K^+ ion with 2Cl^- ions in the same direction. Under physiological circumstances, transport is inward. This process is independent of membrane voltage since the two negative charges from inward movement of 2Cl^- are countered by two positive charges from Na^+ and K^+ . This transporter has been shown to be dependent on the trans-membrane gradient of its respective ions. There have been reports showing that increased levels of intracellular Na^+ and Ca^{2+} have inhibited this transporter (Ikehara et al., 1993, Russell, 2000). It is well known that cardiac myocyte intracellular levels of Na^+ are raised in heart failure; this may have an impact on the correct functioning of the NKCC. The co-transporter is being widely studied at present.

The $\text{Na}^+\text{-H}^+$ exchanger is an important counter-transporter that depends on the trans-membrane Na^+ gradient to transport H^+ ions (produced as by-products of cell metabolism) out of the cell in exchange for Na^+ transported in along its electrochemical gradient. It is an electro-neutral process since the outward movement of one charge by H^+ is counterbalanced directly by the inward movement of a positively charged Na^+ ion. While the Na^+ gradient is a prerequisite for the exchange, $\text{Na}^+\text{-H}^+$ exchange is also regulated by other factors. Activity is stimulated by intracellular acidosis and inhibited by intracellular alkalosis or extracellular acidosis (Vaughan-Jones et al., 1985). The $\text{Na}^+\text{-H}^+$ exchanger also has a vital role to play in the control of intracellular pH. The tight control of pH is extremely important in cell growth, as changes in pH levels often trigger stimulation of mitosis and hypertrophy. The $\text{Na}^+\text{-H}^+$ exchanger operates over a very narrow pH range to maintain intracellular pH at 7.2. It's most important role is the prevention of excessive intracellular acidosis

(Fliegel and Frohlich, 1993, Fliegel, 2008). This is extremely important in the cardiac cell as increasing intracellular acidosis leads to a reduction in contraction and increase in cardiac arrhythmias due to an influx of Na^+ and subsequently Ca^{2+} . Hormonal control is also an important factor in the activation of the $\text{Na}^+\text{-H}^+$ exchanger e.g. by insulin and vasopressin which both stimulate the exchanger. The exchanger is also influenced by platelet derived growth factor and epidermal growth factor (Fliegel and Frohlich, 1993, Fliegel, 2008).

While it is widely appreciated that the trans-membrane electrochemical gradients generated by the $\text{Na}^+\text{-K}^+$ pump are key determinants of cellular ion homeostasis, it is less widely known that the Na^+ gradient also serves in the secondary active transport of organic compounds into cells. Compounds integral to energy metabolism are of particular importance for our understanding of cardiac physiology and pathophysiology. A Na^+ -dependent glucose transporter (GLUT) mediates cellular glucose uptake in several tissues, including the myocardium. There are many isoforms of this enzyme that have been characterized and these isoforms differ in tissue expression, substrate specificity and in their pharmacokinetics (e.g. GLUT-1 is almost universal and uses glucose as its substrate and is Na^+ dependant, while GLUT-11 uses fructose as a substrate and is found specifically in heart and skeletal muscle). The most abundant forms in the myocardium are GLUT 1 and 11 (Abel, 2004, Mann et al., 2003, Doege et al., 2001). GLUT-1 mediated glucose uptake into myocytes may be particularly important in heart failure when there is a shift from lipid-dependent energy generation towards a glucose-dependent metabolism (Abel, 2004).

While glucose is important in cellular ATP synthesis, creatine is important for ATP transport and utilisation within the cells. As creatine is not produced within the cardiac cell, availability of creatine is dependent on the Na⁺-dependent creatine transporter. Creatine produced in the liver and kidney is transported in the bloodstream to the heart where it is transported across a steep concentration gradient up to ~ 50 fold (Guimbal and Kilimann, 1993, Neubauer, 2007). The creatine transported within the cell is used by mitochondrial creatine kinase to form phosphocreatine and ADP from ATP. The diffusion of phosphocreatine to the myofibrils allows the rapid transfer of ATP, as phosphocreatine is catalysed by creatine kinase to reform ATP. Thus any factor that affect the Na⁺ gradient across the cell membrane will effect the energy kinetics of the cell (Neubauer, 2007, Guimbal and Kilimann, 1993, Ten Hove et al., 2005). It follows that while the Na⁺-K⁺ pump is a major ATP-consuming process; it is also ultimately a key determinant of cellular ATP supply and cellular energy metabolism. Since the Na⁺-K⁺ pump determines the intracellular concentration of the ions that ultimately determine cell volume through their osmotic effects, the Na⁺-K⁺ pump also has an important role in the regulation of cell volume especially after osmotic shrinkage (Therien AG, 2000). It may be of particular clinical relevance that intercellular levels of Na⁺ have a direct effect on growth and hypertrophy in both cardiac (Xie Z et al., 1999) and non-cardiac cells (Berk et al., 1990, Rao et al., 1990, Stanton and Kaissling, 1989). In view of the central role the Na⁺-K⁺ pump plays in multiple cellular processes, understanding its regulation is pivotal to our understanding of normal and abnormal cell function.

1.2. Key features of the Na⁺-K⁺ pump

The linkage of ion transport to ATPase activity was first described 50 years ago by the Danish scientist Jens Christian Skou in a seminal 1957 paper (Skou, 1957). Skou was awarded the Nobel Prize for chemistry 40 years later in 1997 for his discovery (Skou, 1998). A vast amount of work has been done to define the structure, reaction cycle and regulation of the Na⁺-K⁺ pump since Skou's 1957 discovery.

1.2.1. Structure of Na⁺-K⁺ pump

Na⁺-K⁺ ATPase is a heterodimeric protein that is embedded in- and spans the membrane of all eukaryotic cells. It consists of α and β subunits in stoichiometric amounts. The α subunit contains the binding sites for ATP, Na⁺, K⁺, cardiac glycosides, specific inhibitors of the enzyme and the phosphorylation site (Glitsch, 2001). This subunit is responsible for the catalytic, pharmacological, and transport characteristics of the ATPase. There have been four tissue specific isoforms of the α subunit identified, α_1 , α_2 , α_3 and α_4 (Shamraj and Lingrel, 1994, Herrera et al., 1987). The α_1 subunit is universal to all tissue types while α_2 is found in nervous and adipose tissue. α_3 is found in nervous system tissue but also in macrophages and the cornea (Huang et al., 2003, Vignery et al., 1991). The α_4 isoform has only been found in male reproductive tissues (Blanco et al., 2000). The Na⁺-K⁺ pump is classified as a member of the P-type ATPases because the α subunit can exist as a phosphorylated

intermediate during its catalytic reactions (Lutsenko and Kaplan, 1995) (discussed further in Chapter 7).

The β subunit is often referred to as a chaperone that helps guide the insertion of the α subunit into the cell membrane. This function may represent the rate-limiting step of the formation of the mature enzyme in the plasma membrane (Mercer et al., 1993). In addition to its function as a chaperone, the β subunit also modulates the transport properties of the ATPase (Geering, 2001). The β subunit is a glycosylated protein that exists in three isoforms, β_1 originally isolated from the kidney (Martin-Vasallo et al., 1989), β_2 and β_3 which are found in a variety of tissues (Malik et al., 1996). The combination of various β and α isoforms can cause a subtle alteration in the tightly controlled pump kinetics with regard to activation by Na^+ , K^+ , ATP, and inhibition by cardiac glycosides. This may confer slightly different tissue specific characteristics on the $\text{Na}^+\text{-K}^+$ pump.

A third subunit, γ , has been described in some tissues, particularly the kidney (Mercer et al., 1993, Sweadner et al., 2003). Subsequent studies showed that the γ subunit is a member of a larger family of proteins referred to as FXYD proteins, named after their consistent amino acid sequence containing the FXYD motif and a conserved glycine and serine residue. These proteins are short single span membrane proteins (<100 amino acids) that have been shown to modulate ion transport (Sweadner and Rael, 2000). The FXYD proteins are expressed in a tissue-specific manner. The γ subunit, expressed in the kidney, known as FXYD2, was the first FXYD protein found to be associated with Na^+ -

K^+ ATPase (Forbush et al., 1978). It has been shown to have multiple effects on the Na^+ - K^+ pump. The FXYD protein expressed in muscle, FXYD1, also known as phospholemman, is unique, in contrast to other FXYD proteins, as it possesses a cytoplasmic terminal with phosphorylation sites accessible to intracellular kinases. As a consequence, it may respond to signaling pathways that activate kinases and thus have a regulatory role in Na^+ - K^+ pump function. The other FXYD proteins can change function when they co-localise with the Na^+ - K^+ pump (Cornelius and Mahmoud, 2003a, Cornelius et al., 2001) but the absence of phosphorylation sites on their cytoplasmic terminal suggest they are unlikely to be responsive to activation of messenger pathways, *i.e.*, they have a modulatory rather than a regulatory role.

The tertiary structure of the Na^+ - K^+ pump molecule in pig renal tissue has recently been definitively established by Morth et al (Morth et al., 2007). The characterisation of the pump structure verified the long held theory that the larger α subunit was similar to the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) ion pump, a member of the P-type ATPase family. The differences in structure are related only to subtle differences in amino acids and the binding of K^+ ions by the Na^+ - K^+ ATPase rather than protons. The revelation of the structure may help to clarify the interactions between the subunits of the pump. Defining the structure revealed direct contact between the α and β subunits at the $\alpha M7$ and $\alpha M10$ positions. These interactions may be important determinants of modulation and regulation of the Na^+ - K^+ pump (Morth et al., 2007, Gadsby, 2007) (for further discussion see Chapter 7).

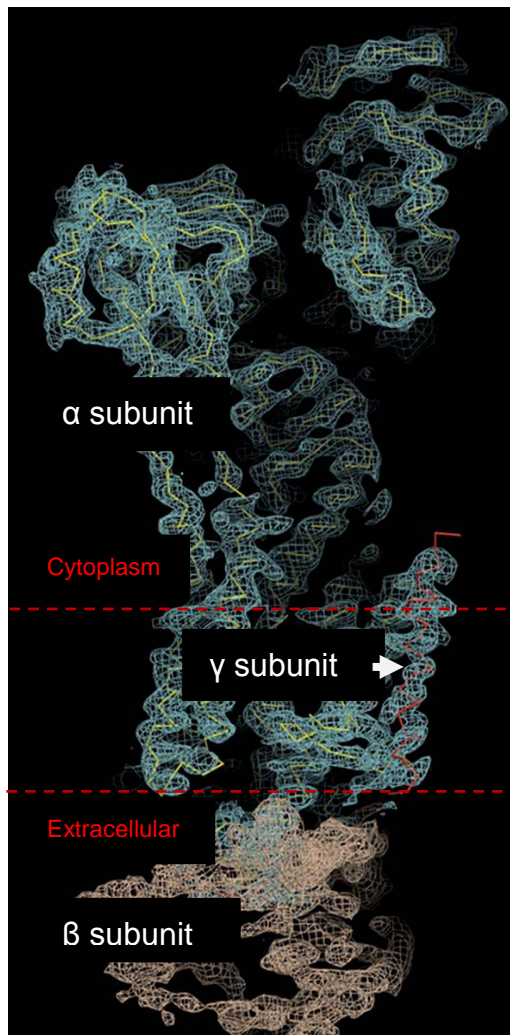


Figure 1.2 The crystalline structure of the Na⁺-K⁺ ATPase

Experimental electron density map of the $\alpha\beta\gamma$ complex demonstrating its intracellular cytoplasmic and extracellular portions.

Adapted from *Crystal structure of the sodium-potassium pump.*

Morth et al, Nature

450(7172)1043-9.

1.2.2. The Na⁺-K⁺ pump cycle

The Albers-Post scheme is an established model of the operation of the Na⁺-K⁺ pump cycle (Karlsh et al., 1978) (See Figure 1.3). According to this scheme, the Na⁺-K⁺ pump can exist in two conformations, E₁ and E₂, with binding sites for transported cations that change orientation according to the conformational state of the enzyme. Phosphorylation/dephosphorylation steps drive the cyclic transitions between the Na⁺ binding (E₁) and the K⁺ binding (E₂) conformations.

According to the Albers–Post model, three intracellular Na^+ (Na_i) bind to the E_1 form of the enzyme, which is then phosphorylated by ATP resulting in the formation of E_1P and Na^+ occlusion within the enzyme. A conformational change then occurs to E_2P and the Na^+ binding sites are exposed to the extracellular surface. The three Na^+ ions are then released followed by binding of two extracellular K^+ ions to the E_2 form of the enzyme. The E_2 form is then dephosphorylated in a step facilitated by allosteric binding of ATP and conformational change back to E_1 occurs. This allows two K^+ to be released into the cytoplasm (See Figure 1.3).

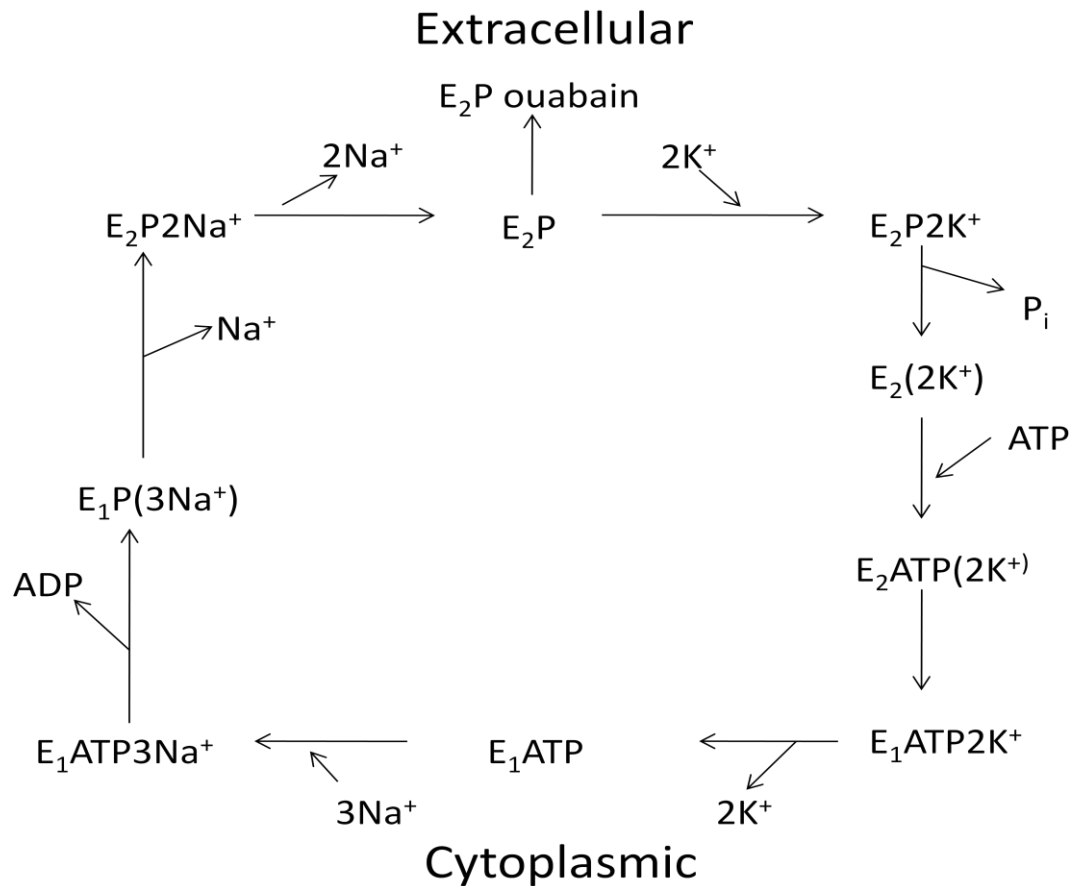


Figure 1.3 The Na⁺-K⁺ pump cycle as described by the Albers-Post reaction scheme

The E₁ conformation has intracellularly facing cation-binding sites and has a high affinity for Na⁺ and ATP. Binding of Na⁺ catalyses the phosphorylation by previously bound ATP creating the E₁P form. Three bound Na⁺ ions are then occluded in the molecule and transported to the extracellular surface. Another conformational change to the E₂ form occurs and the three Na⁺ ions are released in a sequential manner. The E₂P binds two K⁺ ions. This induces dephosphorylation of the pump molecule and subsequent occlusion of K⁺ ions to form E₂(2K). The release of K⁺ at the intracellular surface is catalysed by the binding of ATP. After release of K⁺ the enzyme returns from the E₂ to the E₁ form, ready for a new cycle. (Adapted from Glynn, 1993).

1.3. Regulation of the Na⁺-K⁺pump.

1.3.1. Regulation of the Na⁺-K⁺pump by its ligands, Na⁺, K⁺ and ATP.

The rates of enzymatic reactions are limited by their ligands if they are not present in saturating concentrations. This also applies to the Na⁺-K⁺ pump and its ligands, Na⁺, K⁺ and ATP. Under physiological conditions, intracellular concentrations of ATP are not believed to be rate limiting. In contrast, the intracellular Na⁺ concentration is well below the level where Na⁺ binding saturates, making it a determinant of the overall forward pump rate (rate-limiter). The sensitivity of the Na⁺-K⁺ pump to intracellular Na⁺ concentration varies widely and does not directly reflect binding of Na⁺ to the pump's Na⁺-sensitive E₁ conformational state. Binding to the E₁ conformation occurs in competition with cytosolic K⁺. This K⁺/Na⁺ antagonism is particularly pronounced in cardiac tissue (Therien and Blostein, 1999). In addition, binding of intracellular Na⁺ to the pump is dependent on membrane voltage (Hansen et al., 2002, Rakowski et al., 1997b, Gadsby et al., 1985). It follows that the Na⁺ sensitivity is highly dependent on specific experimental conditions. The intracellular K⁺ concentration and/or membrane voltage have been either unknown or not controlled in most studies looking at the Na⁺ sensitivity of the pump. When these uncertainties are taken into account it is estimated that the Na⁺ concentration for half-maximal pump activation (K_{0.5} mean affinity constant value) in cardiac myocytes is ~20 mM (Glitsch, 2001). Since this is reasonably close to physiological intracellular levels, changes in intracellular Na⁺ can alter pump activity.

While K^+ inhibits the overall forward rate of the pump by competing with Na^+ for binding to the pump's E_1 conformation at intracellular sites, it also activates the pump by binding to the E_2 conformation at extracellular sites. As is the case for binding of Na^+ intracellularly, extracellular binding of K^+ depends on membrane voltage (Peluffo et al., 2004, Peluffo et al., 2000). The $K_{0.5}$ for K^+ reported over the last ~20 years varies widely. With improvements in experimental techniques the range has narrowed and more recent estimates are in the ~1-2 mM range for cardiac myocytes (Glitsch, 2001). At a physiological extracellular K^+ concentration of ~5mM, extracellular K^+ binding is nearly saturated and minor changes in K^+ concentration have little effect on pump rate. However, in states of hypokalaemia, the K^+ concentration can have a significant rate-limiting effect. This accounts in part for the propensity for digitalis toxicity in hypokalaemia.

1.3.2. Voltage dependence of the Na^+ - K^+ pump

The pump rate is dependent on the voltage of the membrane (V_m) (Rakowski et al., 1997a). The major voltage dependent steps can be interpreted in terms of the “access channel” model. According to this, the translocation of charge through the membrane is a direct result of the location of the Na^+ and K^+ binding sites within the electrical field (Lauger, 1991). This model is depicted in Figure 1.4. If the channel consists of a wide opening, which is not very selective and allows many kinds of ions to enter, the channel has a high conductance and there is only a small voltage drop across the channel. However if the channel is

highly selective and has a narrow opening, conductance is low and the voltage drop across the channel is high, resulting in the apparent dissociation constant of the ion becoming voltage dependent. The major steps in the creation of voltage dependence involve the release of Na^+ ions and the binding of the extracellular K^+ ions at external pump site (Sagar and Rakowski, 1994, Gadsby et al., 1989, Apell and Karlish, 2001, Shainskaya et al., 2000). However, as described in the previous section, binding of intracellular Na^+ and extracellular K^+ is also voltage-dependent.

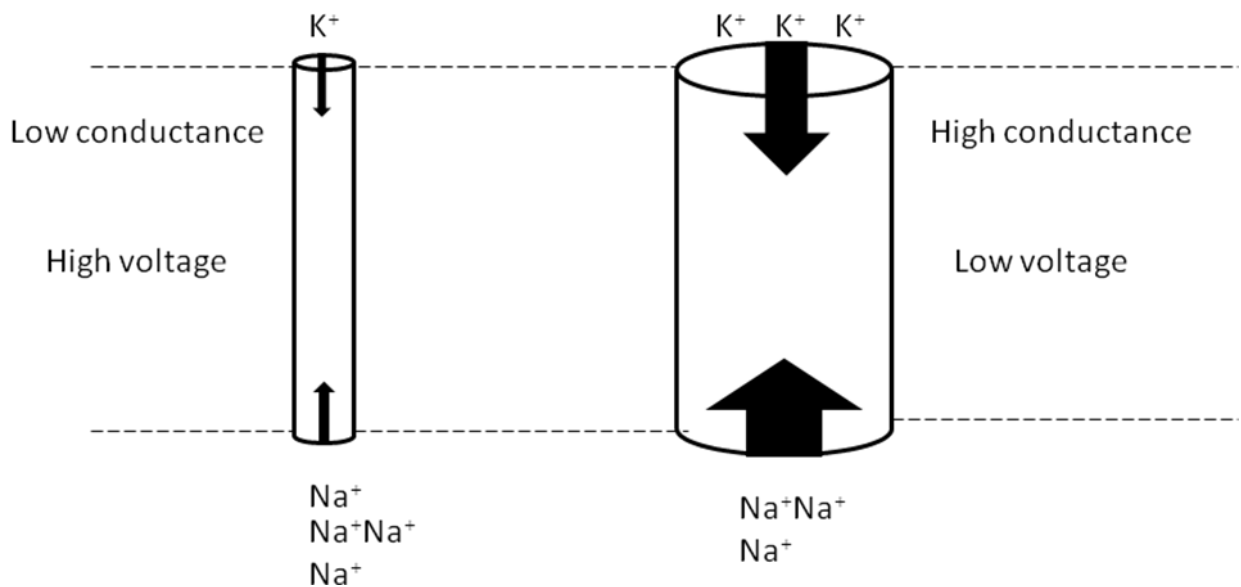


Figure 1.4 Access channel model

Ion access channels in the cell membrane can control the rate of ion entry and exit thereby controlling the voltage and membrane potential. A narrow access channel is highly selective and allows only a small number of ions to cross the membrane. This is low conductance and generates a high voltage across the membrane. However if the channel is wide and non-selective, it has a high conductance and therefore a low voltage across the membrane.

1.4. Na⁺-K⁺ pump regulation: Phosphorylation or Reactive Oxygen Species?

1.4.1. Role of phosphorylation in Na⁺-K⁺ pump regulation.

It is well established that the Na⁺-K⁺ pump is regulated by a variety of hormones and that intracellular messenger molecules play a pivotal role in linking hormone cell surface receptors to the pump molecule. Kinases, in particular protein kinase C (PKC) and protein kinase A (PKA), have been strongly implicated in Na⁺-K⁺ pump regulation. However, there have been significant difficulties with the paradigms that have been proposed. Pump regulation has often been attributed to direct phosphorylation of the Na⁺-K⁺ pump molecule by PKA and/or PKC. However, putative phosphorylation sites in the native pump molecule are very poorly accessible to the kinases (Sweadner and Feschenko, 2001). Indeed, kinase-induced phosphorylation can usually only be demonstrated when the pump molecule is markedly denatured (Cornelius and Mahmmoud, 2003a). In addition, kinase activation is not necessarily associated with phosphorylation of the pump molecule and the phosphorylation that does occur may be to a very low stoichiometry. The physiological role of phosphorylation of the Na⁺-K⁺ pump molecule in its regulation is therefore uncertain (Cornelius et al, 2001). In view of these difficulties it is not surprising that reported functional responses to kinase activation are highly variable: Na⁺-K⁺ pump *stimulation* or *inhibition* attributed to PKA- as well as PKC-mediated phosphorylation are reported with almost identical frequency in numerous published studies (Therien and Blostein, 2000). An alternative paradigm

accounting for hormone- and kinase-mediated pump regulation is much needed.

1.4.2. Potential role of reactive oxygen and nitrogen species.

In view of the difficulties with attributing $\text{Na}^+\text{-K}^+$ pump regulation to phosphorylation of the pump molecule, alternative mechanisms for regulation should be considered. Oxidation may have a role because protein function can be altered by oxidation and nitrosylation of susceptible amino acid residues (Hess et al., 2005, Stadtman and Levine, 2003). Both the $\text{Na}^+\text{-K}^+$ pump subunits and the lipid membrane in which they are embedded contain residues that can be oxidized. In support of a role for oxidation, exposure of $\text{Na}^+\text{-K}^+$ ATPase to oxidants alters its activity (Szabo, 2003, Muriel et al., 2003, Varela et al., 2004) and chemically induced changes in the redox state can alter $\text{Na}^+\text{-K}^+$ pump function in intact cells (Petrushanko et al., 2006). Reactive oxygen species (ROS) are implicated in various cellular signalling cascades (Cohen and Adachi, 2006).

A candidate signaling mechanism should have easy access to regulatory sites of the $\text{Na}^+\text{-K}^+$ pump and, to achieve specificity, signaling needs to be spatially confined. These characteristics may be offered by the oxidizing effect of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) on the sulfhydryl groups of amino acids on the $\text{Na}^+\text{-K}^+$ pump molecule. Thiol groups on cysteine residues are particularly susceptible to oxidation by peroxynitrate

(ONOO⁻). The thiol groups can undergo essentially irreversible sulphinic or sulphonic oxidation, or in the presence of abundant cellular antioxidant tripeptide glutathione, reversible S-glutathionylation. S-glutathionylation confers a negative charge from glutamine that may change protein function and thus have a role in cellular signaling (Cohen and Adachi, 2006). The α subunit of the Na⁺-K⁺ pump has 23 free sulphhydryls and other oxidisable groups that are facing the cytoplasmic environment and are very vulnerable to oxidation and/or nitrosylation by ROS/RNS moieties (Ellis et al., 2003). The β_1 subunit also contains a number of cysteines that are potential candidates for oxidative modification. However, it is not known if ROS participates in physiological signalling regulating the Na⁺-K⁺ pump in the intact cell.

ROS are a family of molecules including molecular oxygen (O₂) and its derivatives produced in aerobic cells (Harrison, 1997). The generation of ROS by endothelial cells is well documented (Harrison, 1997, Somers et al., 2000, Tsutsui, 2004). The ROS family includes superoxide anion (O₂⁻), hydroxyl radical (OH[·]), lipid radicals and hypochlorous acid (HOCL). The superoxide ion can be dismutated to form hydrogen peroxide (H₂O₂) or it can combine with nitric oxide (NO[·]) to form the RNS peroxynitrite (ONOO⁻). ROS and RNS contribute importantly to oxidant stress (Cai and Harrison, 2000, Li and Shah, 2004) and will often be referred to collectively as ROS/RNS in this thesis. Within the mammalian cell, there are many sources of ROS including NADPH oxidase, xanthine oxidase (XO), cytochrome p450 enzyme, arachidonic acid pathway enzymes, lipoxygenase, cyclooxygenase and NO synthase under certain conditions.

Potential stimuli for the generation of $O_2^{\cdot-}$ include pathological factors such as ischemia-reperfusion, high glucose levels and shear stress, at least in endothelial cells. However, the fact that growth factors and the vascular hormones endothelin 1 and angiotensin II (Ang II) also stimulate synthesis of ROS is consistent with a wider role of ROS in signaling in the cell. The role of ROS in the pathogenesis of cardiovascular disease has become appreciated as our knowledge about how ROS mediates cellular responses to pathological stimuli increases. ROS/RNS have been implicated in the pathogenesis of hypertension, atherosclerosis, remodelling after ischemic events and cardiac failure (Romero-Alvira and Roche, 1996, Ohara et al., 1993, Zaugg et al., 2003). See Figure 1.5.

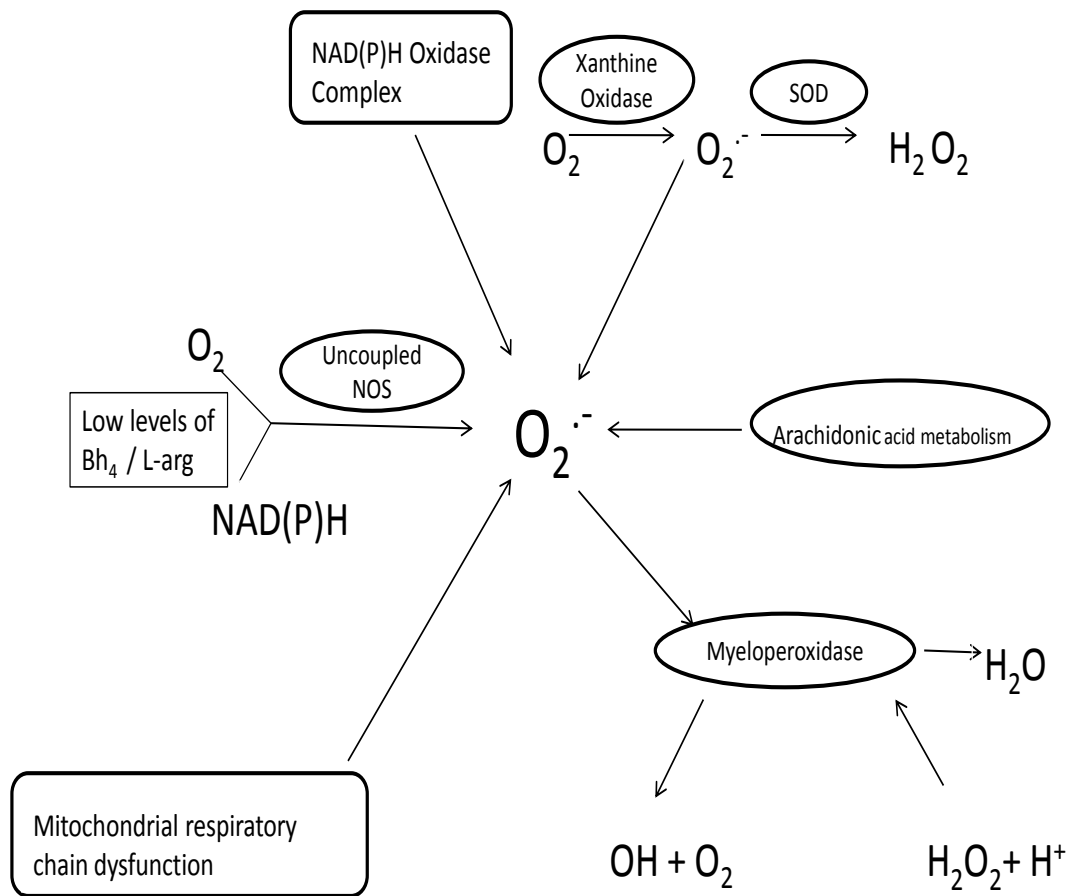


Figure 1.5 Sources of ROS in vascular cells

Activated NADPH oxidase and XO generate superoxide ($O_2^{\cdot-}$). NOS switches from a coupled state to an uncoupled state and generates $O_2^{\cdot-}$ with decreased availability of 5,6,7,8-tetrahydrobiopterin (BH_4) or L-arginine (L-arg). Dysfunctional mitochondrial respiratory chain enzymes are another source of $O_2^{\cdot-}$ generation. SOD isoforms (Mn SOD and CuZn SOD) dismutate $O_2^{\cdot-}$ to produce hydrogen peroxide (H_2O_2). Myeloperoxidase generates H_2O from H_2O_2 . H_2O_2 reacts with transition metals to produce hydroxyl radicals (OH^{\cdot}).

1.4.3. NADPH oxidase.

While NADPH oxidase is not the only source of $O_2^{\cdot-}$ within the cardiac myocytes it is the main source (Griendling et al., 2000). In the context of regulation of the sarcolemmal Na^+K^+ pump, NADPH oxidase is of particular interest because it is membrane-associated. At first sight, NADPH oxidase may seem an unlikely candidate as part of a messenger pathway in the cardiac myocyte since its first identified role, described in phagocytes, involved the production and extracellular secretion of $O_2^{\cdot-}$ to mediate destruction of invading bacteria. However, it is now widely appreciated that functional NADPH oxidase is expressed in a variety of tissues, including the tissues of the cardiovascular system (Cai et al., 2003, Griendling et al., 2000, Szabo, 2003) and that $O_2^{\cdot-}$ can be secreted intracellularly (Griendling et al., 2000). The latter is of particular importance for a role of $O_2^{\cdot-}$ in cellular signaling.

The functional properties of NADPH oxidase are closely linked to its structure. The enzyme is a multi-subunit complex that consists of a membrane-bound flavocytochrome made up of the subunits $p22^{phox}$ which is a 22-kDa α subunit and one of several catalytic non phagocytic NADPH oxidase (Nox) isoforms. Of the five distinct Nox isoforms identified to date, the major isoforms expressed in cardiomyocytes are $gp91^{phox}$ (Nox2) and Nox4 (Murdoch et al., 2006, Griendling et al., 2000). Nox2 is regulated by 4 cytosolic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac (low molecular weight GTP binding protein), which translocate to the membrane on enzyme activation and associate with the subunits that are already membrane-bound. Translocation of the cytosolic subunits, and hence formation of a functional activated NADPH oxidase complex, is dependent upon

phosphorylation of the p47^{phox} cytosolic subunit. Interestingly there is evidence that Nox4 is continuously active and does not require a cytoplasmic subunit to increase its activity. See Figure 1.6.

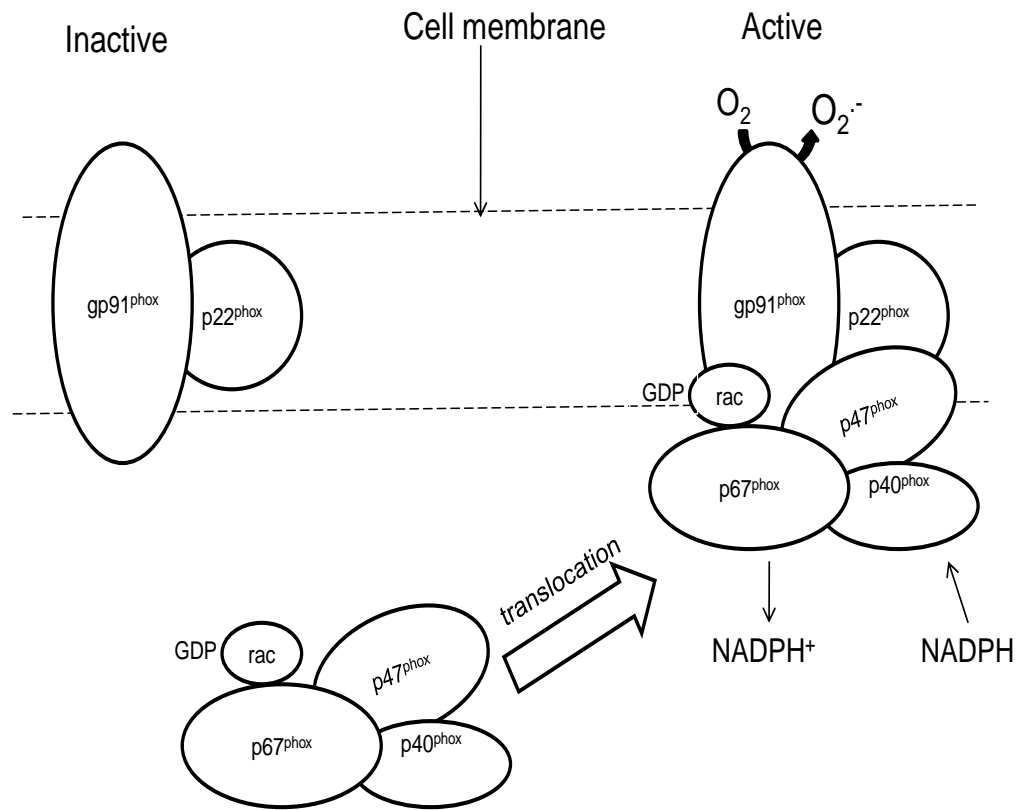


Figure 1.6. Structure and activation of NADPH oxidase

Activation of NADPH oxidase involves translocation of the cytosolic subunits p47^{phox}, p67^{phox}, p40^{phox} and Rac to the membrane where they bind to cytochrome b_{558} . The cytochrome b_{558} is composed of p22^{phox} and gp91^{phox} (Nox2) subunits. The production of NADPH⁺ and O₂⁻ cannot proceed until this translocation is complete.

1.4.4. Nitric oxide synthase.

The RNS, nitric oxide (NO), is also of considerable interest as a regulator of Na⁺-K⁺ pump activity. It has been reported that exogenous NO supplied by the pharmacological donor compound sodium nitroprusside stimulates the Na⁺-K⁺ pump in cardiac myocytes (William et al., 2005a). However, NO can also be generated endogenously by nitric oxide synthase (NOS). There are three isoforms, NOS1, NOS2 and NOS3. They are also known as “neuronal” (nNOS), “inducible” (iNOS) and “endothelial” (eNOS) nitric oxide synthase (Alderton WK et al., 2001). nNOS is mainly found in central and peripheral nerve cells but it is also found within the cardiac myocyte. iNOS was first found in murine macrophages but has since been identified in many cell types. eNOS is expressed in endothelial, endocardial and, at a much lower concentration, in the cardiac myocyte (Shah and MacCarthy, 2000, Balligand et al., 1994). Of these, eNOS is the only NOS that is associated with the sarcolemmal membrane.

NOS utilises L-arginine and molecular oxygen as substrates. Co-factors required for the production of NO include; reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R)-5,6,7,8- tetrahydrobiopterin (BH₄) (Stuehr et al., 1991, Mayer et al., 1991, Hevel and Marletta, 1992). NADPH acts as a proton donor while BH₄ donates an electron to a haem-dioxy intermediate during the NOS oxygen cascade (Li and Shah, 2004). All of the isoforms of NOS are flavoenzymes; complex homodimeric oxidoreductases that shuttle

electrons from the reductase domain of one monomer (containing the co-factors FAD, FMN, NADPH) to the oxidase domain of the other subunit containing the active heme site (Matsuda and Iyanagi, 1999). Figure 1.7 shows key functional properties of NOS.

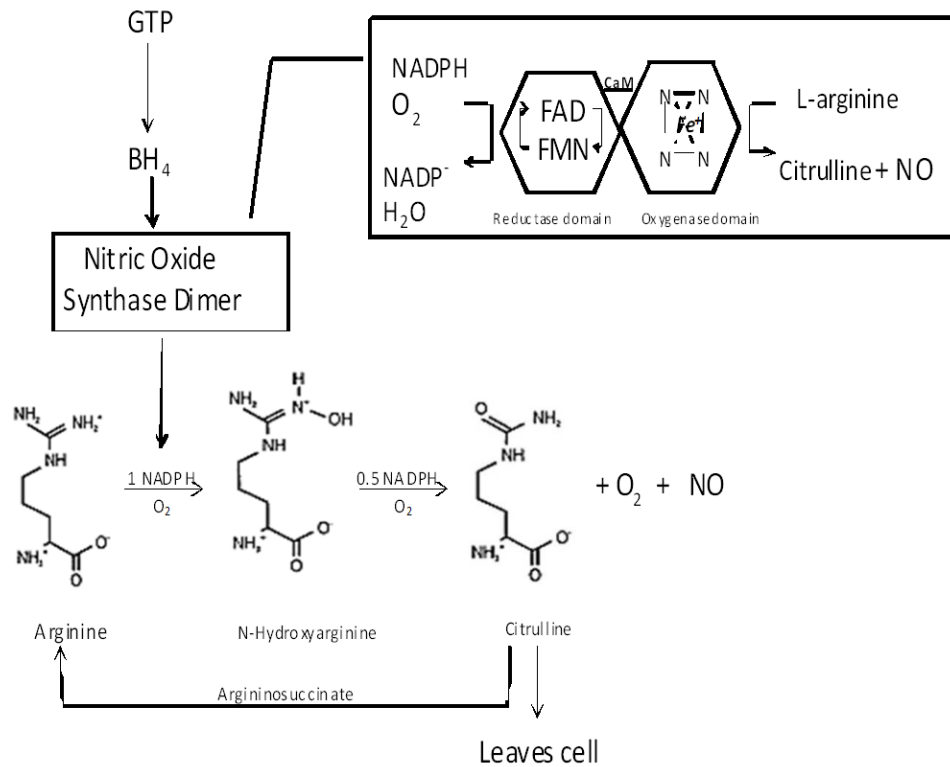


Figure 1.7 Structure and interactions of Nitric Oxide Synthase

Nitric oxide synthase is a dimer that is closely associated with calmodulin. NOS is tightly bound to its co-factors tetrahydrobiopterin (BH₄), FAD, FMN and a haem moiety. Electrons are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and BH₄ at the active site to catalyse the reaction of oxygen with L-arg, generating citrulline and NO as products. The majority of citrulline leaves the cell, however it can be converted back to L-arg by the enzyme arginosuccinate.

A potential role of endogenous NO generated by NOS in Na⁺-K⁺ pump regulation is highlighted by the fact that eNOS co-localises with the pump in membrane caveolae in cardiac myocytes (Liu et al., 2003) and by the previous demonstration of pump stimulation induced by exogenous NO (William et al., 2005a). However, effects of NO can be highly compartmentalized in molecular microdomains (Hess et al., 2005) and results obtained using a pharmacological NO donor may be critically different from effects of endogenously synthesised NO (Massion PB, 2003 , Dudzinski et al., 2006). It cannot be assumed that the effect of endogenously synthesised NO is similar to the effect of NO sourced from a pharmacological donor.

While it is reasonable to think that endogenous NO synthesized by NOS may stimulate the Na⁺-K⁺ pump, effects of NO production in an intact myocyte are likely to be complex because NO readily interacts with O₂⁻ to form the RNS peroxynitrite (ONOO⁻). This product is a particularly good candidate as a regulator of the Na⁺-K⁺ pump because its acid form is highly lipid soluble and hence membrane permeable, while the charged O₂⁻ species is expected to be strictly confined to the microdomain where it is formed. It may therefore not have easy access to important regulatory sites of the highly lipophilic Na⁺-K⁺ pump molecule.

The schematic diagrams shown in Figures 1.5 and 1.7 imply that synthesis of O₂⁻ and NO are mediated by enzymes working independently of each other,

however, in reality there can be a complex interaction. The highly reactive ONOO^- can oxidise the NOS cofactor BH_4 to BH_2 . As a consequence, the electron transfer sequence in the NO syntheses is interrupted and the electron is diverted to molecular oxygen and hence formation of O_2^- . This functional change in NOS is referred to as “uncoupling”. Uncoupling of NOS can also occur when there is a deficiency in its substrate, L-arginine (Griendling et al., 2000, Li and Shah, 2004). The functional interaction between NOS and NADPH oxidase is illustrated in Figure 1.8.

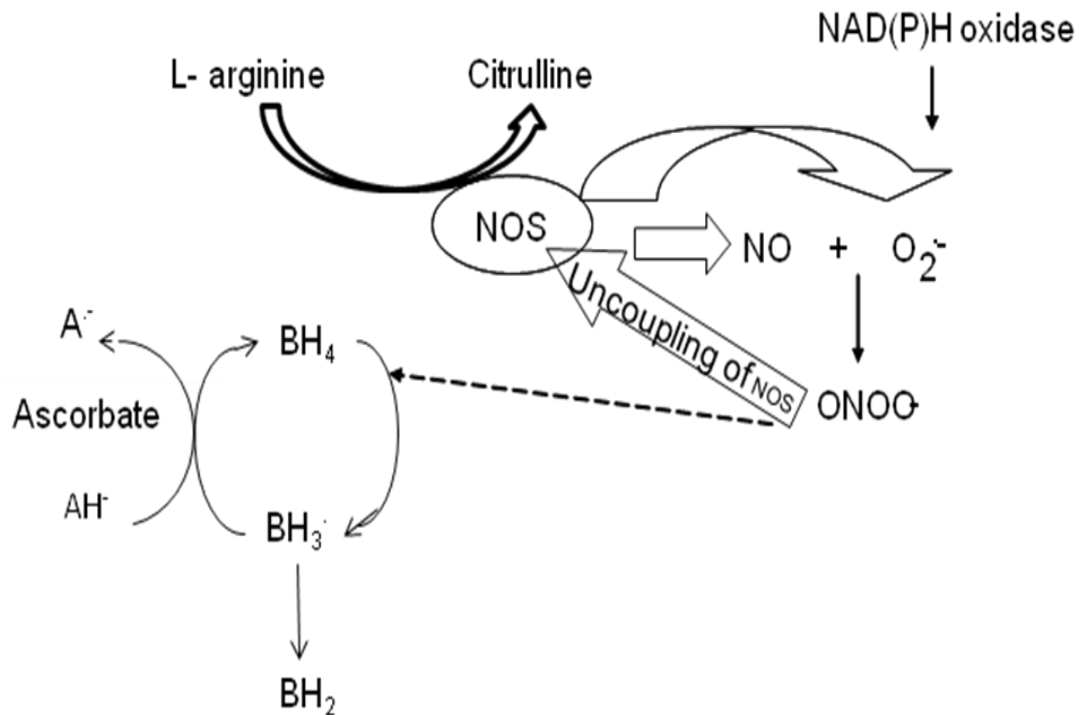


Figure 1.8 Schematic representation of Nitric Oxide Synthase (NOS) uncoupling

As illustrated, nitric oxide (NO) is generated by NOS using L-arginine (L-arg) as a substrate, with citrulline as a by-product. Co-factors required for this process include BH_4 , ascorbate and molecular O_2 . NO produced by NOS rapidly reacts with superoxide ($O_2^{\cdot-}$) produced by NADPH oxidase to form peroxynitrate ($ONOO^-$). $ONOO^-$ is a highly reactive molecule which can cause uncoupling of NOS and the generation of $O_2^{\cdot-}$ via the oxidation of BH_4 to BH_2 thus rendering it inactive and interrupting the flow of electrons within the enzyme.

1.4.5. Metabolic Pathways for $O_2^{\cdot-}$

The properties and metabolism of the different ROS/RNS species is expected to be important for their utility in signaling. The charged $O_2^{\cdot-}$ molecule is expected to be spatially restricted to the microdomain where it is secreted in a cell. However, the $ONOO^-$ that is formed when $O_2^{\cdot-}$ combines with NO, readily merges with H^+ to form peroxynitrous acid. Peroxynitrous acid can cross membranes to reach other cellular compartments. Superoxide can also be metabolized to another membrane-permeable species, hydrogen peroxide in the “dismutation reaction”, catalysed by superoxide dismutase (SOD). In addition, hydroperoxyl radical and hydroxyl radicals can be formed in the metabolism of $O_2^{\cdot-}$. The various pathways for $O_2^{\cdot-}$ metabolism are summarised in Figure 1.9.

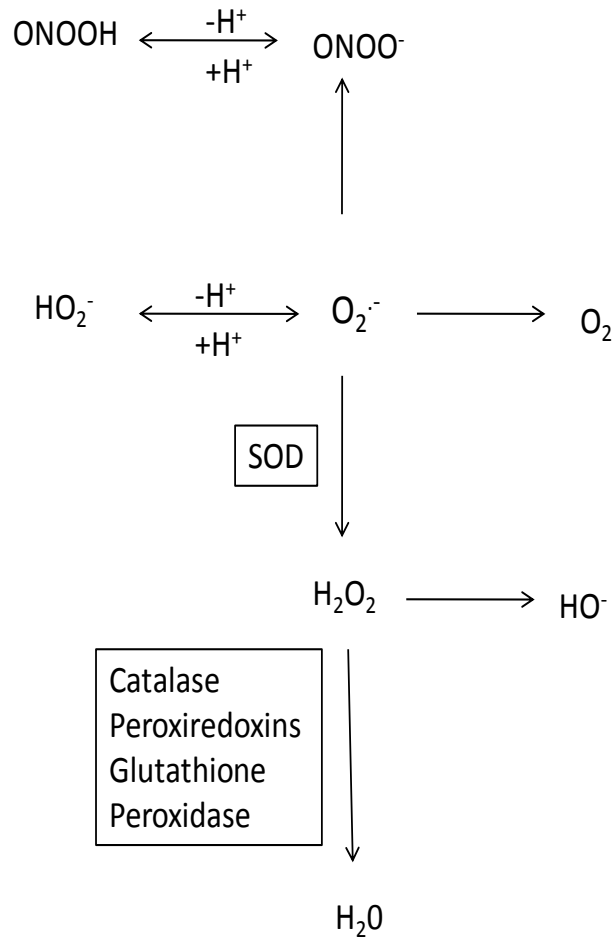


Figure 1.9 Role of superoxide dismutase (SOD) in the reactive oxygen species (ROS) scavenging pathway

SOD catalyzes the reduction of superoxide anions to hydrogen peroxide (H_2O_2). H_2O_2 is then converted H_2O and O_2 by a series of enzymatic reactions. Under normal conditions, ROS are cleared from the cell by the action of SOD, catalase, or glutathione peroxidase.

1.4.6. Superoxide Dismutase.

As indicated in Figure 1.9, superoxide dismutase (SOD) has an important role in the regulation of $O_2^{\cdot-}$ and its derivative metabolites. The concentration of SOD is 10^6 times higher than $O_2^{\cdot-}$ (Johnson and Giulivi, 2005). It converts $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2). To deal with excess of $O_2^{\cdot-}$ produced by uncoupled NOS and/or NADPH oxidase, SOD converts it to H_2O_2 . H_2O_2 in turn is subsequently converted to H_2O and O_2 by catalase and peroxidases. SOD exists in different isoforms. The “cytosolic” (SOD1) and “extracellular” (SOD3) isoforms have zinc and copper at their active sites (Cu-ZnSOD), while the “mitochondrial” (SOD2) isoform contains manganese (MnSOD). The copper acts by undergoing alternate oxidation and reduction to dismutate the $O_2^{\cdot-}$, whereas zinc acts to stabilize the enzyme (Mathers et al., 2004, Johnson and Giulivi, 2005). The rate of reaction of SOD with $O_2^{\cdot-}$ is $\sim 2 \times 10^9 \text{ mol}^{-1}\cdot\text{s}^{-1}$. However the rate of reaction of NO to $O_2^{\cdot-}$ is even faster at $\sim 7 \times 10^9 \text{ mol}^{-1}\cdot\text{s}^{-1}$. As a consequence of these reaction rates, NO may out-compete SOD for $O_2^{\cdot-}$ and generate $ONOO^-$ when NO levels are high. This leads to a reduction in the activity of NO and thereby controlling the level of physiologically available NO. Cu-ZnSOD will be used as an experimental tool in the work presented in this thesis.

1.5. The Renin-Angiotensin system, adrenergic signaling and the Na⁺-K⁺ pump: A role for ROS/RNS in signalling?

The renin-angiotensin system (RAS) was originally mainly of interest in the context of blood pressure regulation. However, more recently it has also been recognized that it plays a major role in the regulation of cardiac function. This has had the practical therapeutic application of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blocking (ARB) drugs becoming established in the treatment of heart failure (Klein et al., 2003). The beneficial effect of ACE-inhibitors and ARBs may well be related to their effect on cardiac myocyte Na⁺ levels.

Raised cardiac myocyte levels of Na⁺ are believed to be important in the electro-mechanical phenotype of impaired contractility and cardiac arrhythmias in heart failure (Pieske B et al., 2002, Pieske and Houser, 2003, Pieske et al., 2003, Bers et al., 2003, Despa et al., 2002, Pogwizd SM et al., 2003). Since treatment of heart failure with ACE-inhibitors and ARBs enhance cellular export of Na⁺ by up-regulating Na⁺-K⁺ pump function (Hool LC et al., 1996, Hool et al., 1995a), it is reasonable to think that the clinical efficacy of the drugs are related to their effect on cell Na⁺ levels. Previous studies have shown that the effect of angiotensin II (Ang II) on the cardiac myocyte Na⁺-K⁺ pump is mediated by activation of PKC (Buhagiar et al., 2001a, Buhagiar et al., 1999, Hool et al., 1995a). However, as described above, it is poorly understood how protein kinases, including PKC, affect the Na⁺-K⁺ pump. Since ROS/RNS are

implicated in the pathogenesis of heart failure (Maack et al., 2003) it is of particular interest that Ang II, *via* the AT₁ receptor (Plumb et al., 2005), is known to stimulate NADPH oxidase, the main source of superoxide in the cardiac myocyte. The possible role of NADPH oxidase in mediating effects of Ang II on the cardiac myocyte Na⁺-K⁺ pump will be explored in the work presented in this thesis.

Regulation of the Na⁺-K⁺ pump by Ang II is of particular interest because of the well-established role of ACE-inhibitors and ARBs in the treatment of heart failure. However, an understanding of the effect of adrenergic signaling on the pump is equally important because of the proven efficacy of β-adrenergic receptor blockade. β₁ and β₂ adrenergic receptors are coupled to adenylyl cyclase (AC), that on stimulation of the receptors, catalyses the intracellular synthesis of cyclic adenosine 3',5'-monophosphate (cAMP) (Wenzel-Seifert et al., 2002). Cyclic AMP in turn activates cAMP-activated protein kinase (PKA). The effect of PKA on the Na⁺-K⁺ pump has been extensively investigated. As referred to above, no consistent pattern of stimulation vs. inhibition has emerged when many different tissue types are compared (Therien and Blostein, 2000). However, in cardiac myocytes, two recent studies have both reported that activation of adrenergic signaling stimulates the Na⁺-K⁺ pump (Despa et al., 2005, Silverman et al., 2005). One might therefore expect that blockade of β₁/β₂ receptors would have the opposite effect, *i.e.* reduce Na⁺-K⁺ pump activity and hence cause an increase in intracellular Na⁺ levels. If raised cytosolic Na⁺ levels play pivotal adverse roles in the pathogenesis of heart failure (Bers et al., 2003, Pieske et al., 2003, Pogwizd SM et al., 2003) it is difficult to reconcile the

beneficial effect of β blockers in its treatment if they promote a further increase in intracellular Na^+ levels. The work presented in this thesis will re-examine the effect of adrenergic signaling on the $\text{Na}^+\text{-K}^+$ pump in cardiac myocytes. Because it is very unlikely that PKA has a direct regulatory effect on the pump (Sweadner and Feschenko, 2001), a potential role of ROS/RNS will be explored.

1.5.1. Angiotensin II.

Angiotensin II (Ang II) is a peptide hormone produced by the renin angiotensin system. It originates as Angiotensin I (Ang I), which is produced from angiotensinogen by renin. (See Figure 1.10.) The juxtaglomerular apparatus in the nephron makes renin. Ang I is converted to Ang II by angiotensin converting enzyme (ACE) of which large amounts are found in the lungs. Not only does Ang II act as a hormone, it also has a paracrine and autocrine role to play. It can be produced by the endothelium in all organs and even cardiac myocytes possess the complete set of metabolic machinery that allows synthesis of the peptide (Leung, 2004)

Ang II binds to two cell receptors, AT_1 and AT_2 . The majority of the hormone's biological effects occur after its activation of the AT_1 receptor. Through this receptor, Ang II induces the expression of a number of growth factors and metalloproteinases, which are all involved in vascular remodelling. Ang II

activates the epsilon isoform of protein kinase C (ϵ PKC) through the AT₁ receptor, which in turn activates NADPH oxidase (Plumb et al., 2005) and hence produce superoxide. It is of particular interest for the work in this thesis that ϵ PKC has been reported to mediate an inhibitory effect on the Na⁺-K⁺ pump in ventricular cardiac myocytes (Buhagiar et al., 2001a) and that PKC activates NADPH oxidase by phosphorylating its p67^{phox} subunit (Zhao et al., 2005). Conversely, blockade of the AT₁ receptor reduces the superoxide production (Warnholtz et al., 1999, Lemay et al., 2000).

The AT₂ receptor is usually expressed in low density in adults; however, its expression is upregulated following vascular injury, cardiac failure, cardiac hypertrophy or salt depletion. The AT₂ receptor opposes the effects of the AT₁ receptor. Stimulation of the AT₂ receptor activates the bradykinin/NOS system and increases the production of NO (Siragy and Carey, 1997). There appears to be “cross talk” between the AT₁ and AT₂ receptors at the biochemical level with the two receptors promoting synthesis of O₂⁻ and NO respectively (de Gasparo and Siragy, 1999). At cell and tissue levels, stimulation of AT₁ causes hypertrophy, angiogenesis, vasoconstriction, interstitial fibrosis and cardiac remodelling while stimulation of AT₂ causes antiproliferation, antiangiogenesis, vasodilatation, a reduction in neointimal formation and inhibition of cardiac remodelling (de Gasparo and Siragy, 1999, Morgan T, 2003, Siragy and Carey, 1997). See Figure 1.10.

Intracellular effects of Ang II are often attributed to an increase in cytosolic Ca²⁺ levels induced by the peptide. However, it is also widely recognised that Ang II

increases oxidative stress by activating NADPH oxidase. This is usually viewed as an adverse effect and independent of effects on cellular ion homeostasis. However, since the $\text{Na}^+\text{-K}^+$ pump, (*via* the $\text{Na}^+\text{-Ca}^{2+}$ exchanger), is a key determinant of intracellular Ca^{2+} , it is reasonable to think that effects of Ang II on ROS and ion homeostasis are closely interrelated.

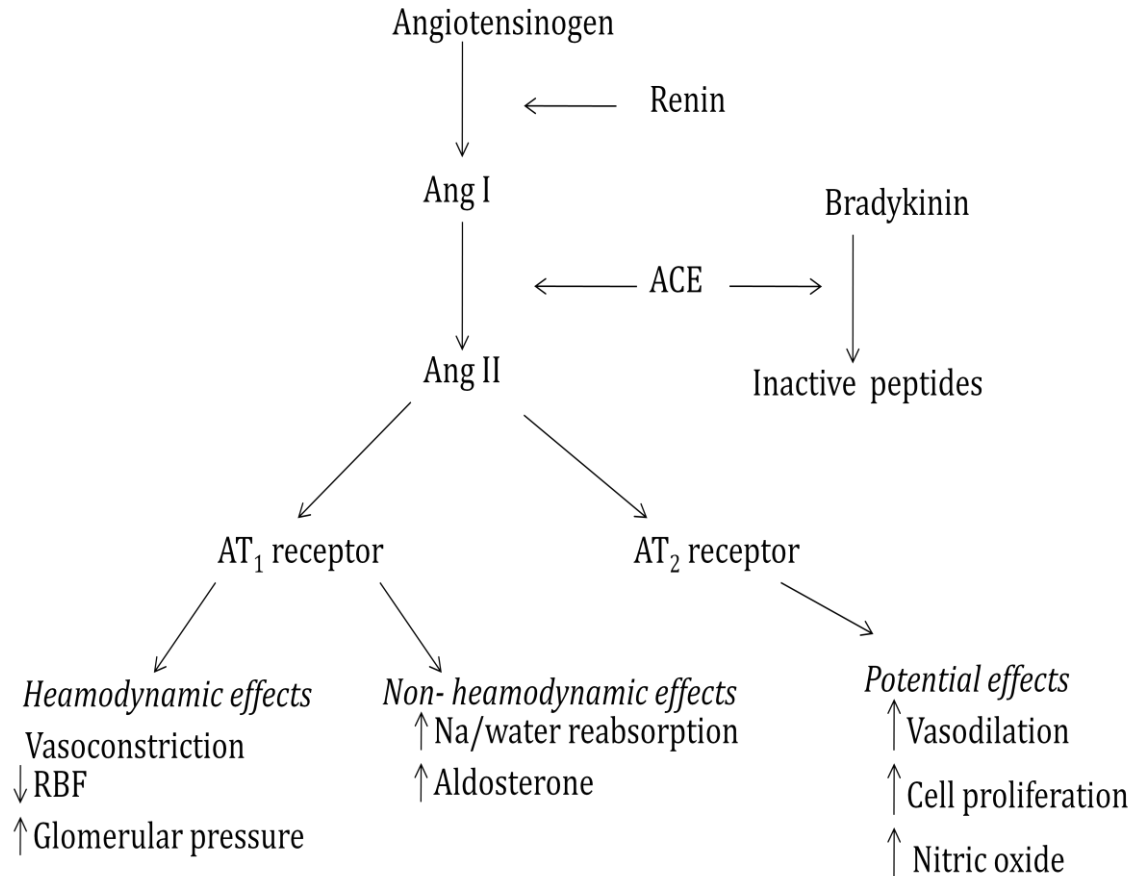


Figure 1.10 Angiotensin II and its receptor activation

The pro-hormone angiotensinogen is cleaved by renin to produce angiotensin I (Ang I). Ang I is converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang II is the active hormone. Ang II binds to the AT₁ and AT₂ receptor to cause its downstream effects. Activation of the AT₁ receptor causes both hemodynamic effects such as a reduction in renal blood flow (RBF), an increase in glomerular pressure and vasoconstriction and non-haemodynamic effects like increasing Na⁺ and water reabsorption and increasing aldosterone production. Activation of AT₂ has the potential to cause an increase in the production of Nitric oxide, hence vasodilation and increased cell proliferation.

1.5.2. β -Adrenergic signalling.

The β -adrenergic system has been implicated in Na^+ - K^+ pump regulation in the heart (Charpentier et al., 1996). Activation of β_1 and β_2 receptors causes an increase in the levels of NO and cAMP which activates PKA. PKA can phosphorylate the p47^{phox} cytosolic subunit of NADPH oxidase *in vitro*, albeit less effectively than PKC (Cheng et al., 1997). However, the phosphorylation does not appear to activate synthesis of $\text{O}_2^{\cdot-}$ in a non-cellular system (Babior, 2000). In contrast to the β_1 and β_2 adrenergic receptors, β_3 receptors are coupled to activation of NOS (Balligand, 2000) in cardiac myocytes. A preliminary report from our laboratory indicates that activation of the β_3 receptors induces Na^+ - K^+ pump stimulation *via* a mechanism dependent on activation of NOS and NO activated guanylyl cyclase (Bundgaard H, 2006). These findings appear to be at odds with previous reports that suggest β -adrenergic Na^+ - K^+ pump stimulation is mediated by a β_1/β_2 adrenergic receptor/cAMP/PKA dependent pathway (Silverman et al., 2005, Fuller et al., 2004, Despa et al., 2005).

In view of the discrepancy, the effect of β_1/β_2 adrenergic receptor activation on the sarcolemmal Na^+ - K^+ pump will be re-examined. Since no ligands for β adrenergic receptors exhibit perfect selectivity for β_1/β_2 vs. β_3 receptors, a compound that selectively activates the adenylyl cyclase coupled to β_1/β_2 receptors rather than a catecholamine will be used in the studies. Since direct phosphorylation of the Na^+ - K^+ pump molecule is unlikely to account for pump regulation, the possibility that ROS/RNS are involved in cAMP/PKA dependent pump regulation in an intact cellular system will also be examined.

1.6. Scope of thesis.

Sections 1.1 to 1.5 have briefly outlined structure and function of the $\text{Na}^+\text{-K}^+$ pump. It has been described how its regulation by hormones and the intracellular messenger pathways coupled to their membrane receptors is poorly understood. Specifically it has described how the hypothesis that direct phosphorylation of the $\text{Na}^+\text{-K}^+$ pump molecule is able to regulate its activity is untenable.

It is already established that ROS/RNS can effect the activity of the isolated enzymatic equivalent of the pump, $\text{Na}^+\text{-K}^+$ ATPase and that ROS/RNS can alter pump activity under pathological conditions. In this thesis the idea that synthesis of ROS/RNS, coupled to hormone receptors and their intracellular messenger pathways, can regulate $\text{Na}^+\text{-K}^+$ pump activity in cardiac myocytes will be examined.

Specifically the thesis will examine if:

(1) Endogenous NOS-mediated synthesis of NO can stimulate the $\text{Na}^+\text{-K}^+$ pump in a manner similar to that reported for exogenous NO supplied by pharmacological donor compounds.

(2) A pharmacological intervention known to uncouple NOS to produce O_2^- causes $\text{Na}^+\text{-K}^+$ pump inhibition.

(3) A hormone ligand known to activate NADPH oxidase (Ang II) causes Na⁺-K⁺ pump inhibition attributable to O₂⁻.

(4) Re-examine the role of β₁/β₂ adrenergic receptor activation in the regulation of the sarcolemmal Na⁺-K⁺ pump and determine if ROS/RNS is involved in the regulation.

Chapter 2

Methods

2.1. Animals and housing.

White male New Zealand rabbits (*Oryctolagus cuniculus*) weighing 2.5–3.0 kg were used for all experiments. The rabbits were housed at the Gore Hill Research Laboratories at Royal North Shore Hospital (RNSH). All were kept in individual cages and the rooms were maintained at a constant temperature of 22 ± 2 °C and 50 ± 5 % humidity with 12 hour light and dark cycles. They were given standard commercial rabbit chow and water *ad libitum*. All animal treatment protocols were approved by the RNSH/University of Technology, Sydney Animal Care and Ethics Committee. The rabbits did not receive any drug treatment or have any manipulation of their feed.

2.2. Isolation of Cardiac Myocytes.

The method used for isolation was adapted from Haddad *et al* (1988). The rabbits were anaesthetised with 50mg/kg ketamine (Parnell Laboratories (Aust) PTY. Ltd.) and 20mg/kg xylazine (Troy Laboratories, PTY, Ltd, NSW, AU) intramuscularly. Heparin (1000U) was given intravenously via a marginal vein in the ear using a 23 G butterfly needle. Once deep anaesthesia was achieved, tested by the rabbits lack of response to painful stimulus(squeezing the paw),

a left lateral intercostal incision at the level of the fifth intercostal space was made. This incision was extended using scissors across the midline and superiorly along the right ribs. The heart was exposed, freed from its attachments and the aorta transected rapidly.

The excised heart was rinsed three times in ice-cold (2-4 °C) Krebs solution containing (in mM): 130 NaCl, 2.8 KCl, 25.0 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 glucose, 19.9 taurine. The pH was adjusted to 7.4. The solution was oxygenated using carbogen (95% O₂ - 5% CO₂) for one hour. Once free from any remaining blood and debris, the heart was attached to a Langendorff perfusion apparatus (Laboratory Supply Sydney, Australia) by securing the aorta to a glass cannula with a silk suture. The cannula tip was positioned 3-4 mm above the aortic valve to ensure adequate perfusion of the coronary arteries. The heart was perfused with cold Krebs solution for 6 minutes. The perfusion solution was then switched to a warm Krebs solution (35°C). The solution was warmed using a thermostatically controlled water bath (Julabo, Seelbach, Germany) and kept at a constant temperature using the recirculation circuit of heated distilled water surrounding the perfusion column. Perfusion of the warm Krebs solution was achieved using a rate adjustable peristaltic infusion pump (Cole-Palmer Instrument Co, Chicago, USA). A glass water jacket, also heated by the recirculation circuit, was placed around the heart. The suspended heart was perfused with warm Krebs solution for 6 minutes, and subsequently with a warmed Krebs solution containing ≈ 217 U/ml of type II collagenase (Worthington Biochemical Corp, Freehold, NJ, USA) and ≈ 874 U/ml of hyaluronidase (Worthington Biochemical Corp, Freehold, NJ, USA). (The ratio of collagenase to hyaluronidase was dependant on the time of year

as rabbit hearts contain more collagen in the winter (Helin and Lorenzen, 1976). The heart was perfused with the enzyme containing solution from 8-14 minutes. The enzymatic digestion of the heart was judged complete when it became enlarged and appeared glassy and translucent. It was cut down from the apparatus using an incision separating the atria from the ventricles; only the ventricles were used in the subsequent experiments.

The ventricles were placed in a beaker containing a small amount (20 mL) of enzyme containing Krebs and coarsely minced using scissors. The cell suspension was then strained through nylon mesh (500 μ M pores) to eliminate large debris. The filtrate was transferred to test tubes and centrifuged for 1 minute 45 seconds at 1500 rpm in Krebs solution. The supernatant was then aspirated and the pellet was re-suspended. This solution was then centrifuged again and the resulting supernatant was then aspirated and the pellet re-suspended in a solution containing equal volumes of Ca²⁺ containing Krebs and Ca²⁺ free Krebs. This solution was centrifuged again for 1 minute and 45 seconds at 1500 rpm. The resultant pellet was then stored in a solution of Ca²⁺ containing Krebs solution at room temperature until experimentation. Myocytes isolated were used for experimentation on the day of isolation only.

2.3. The Whole Cell Patch Clamping Technique.

The patch clamp technique allows the study of the electrophysiological properties of a cell or membrane. It allows control of both the intracellular and extracellular environment as well as control of the membrane voltage (V_m) and measurement of membrane currents. A wide tipped glass pipette is placed on the surface of the cell membrane to form a seal with it. Gentle suction is then applied to rupture the membrane and allow perfusion of the intracellular compartment of the cell with the contents of the pipette. The pipette contains an Ag/AgCl electrode which acts as an interface between the pipette solution and the electronic circuitry. See Figure 2.1.

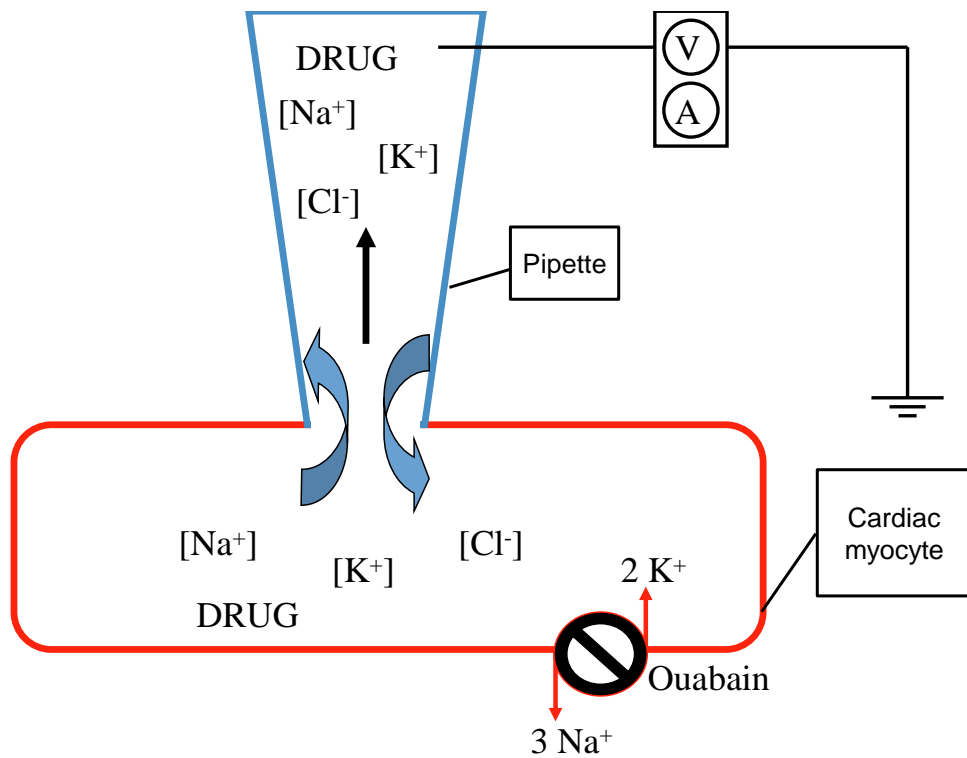
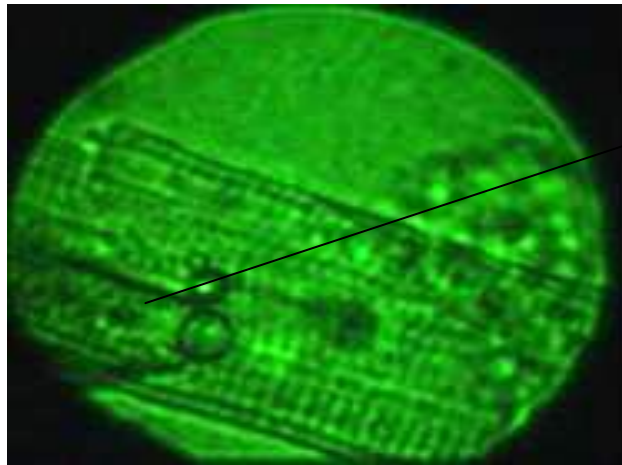


Figure 2.1 The whole-cell patch-clamp technique

A wide-tipped ($\sim 5 \mu m$) pipette is gently placed onto the surface membrane of a single ventricular myocyte. Gentle suction is then applied to the back of the pipette until the cell membrane is ruptured. This allows the patch pipette solution to perfuse the intracellular compartment of the cell and thus control ionic concentrations and membrane voltage. The technique also allows the measurement of membrane currents. The electrogenic Na^+-K^+ pump, the activity of which was measured throughout this study, is represented in the cell membrane.



Glass pipette
on cardiac
myocyte

2.3.1 Tissue bath and experimental set up

A 350 μL tissue bath was secured to the stage of an inverted phase contrast microscope (Nikon TE200, Nikon Corp, Tokyo, Japan), allowing observation of the isolated cardiac myocytes. A peltier controlled heat exchanger warmed the superfusate solutions before they entered the tissue bath. The temperature was maintained at $35\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ via a negative feedback loop (Department of Biomedical Engineering, RNSH). Temperatures were monitored using a telethermometer (Yellow Springs Instruments Co Inc, Yellow Springs, Ohio, USA).

The tissue bath was gravity fed; all superfusates were administered using intravenous infusion sets. Solutions were drained from the tissue bath, under continuous suction. The depth of the tissue bath was maintained at a constant level by the use of a Perspex cap bridge, which separated the tissue bath from a second reservoir. Flow rates of the superfusates were regulated to maintain a constant temperature and a six-way Teflon rotary valve (Redone Inc., Cotati, CA, USA), placed just before the entrance to the bath allowed rapid exchange of the superfusates. All of the equipment was placed on a vibration isolation

table (Technical Manufacturing Corp, MA, USA) located inside a Faraday cage. The bath solution was grounded via an Ag/AgCl electrode placed downstream in the tissue bath. Prior to experimentation, the ground electrode and tip of the filled patch pipette were lowered in to the tissue bath and the potential offset was set to zero. All metallic equipment was grounded to a point within the Faraday cage to reduce electrical interference and noise.

2.3.2. Patch clamp equipment.

Wide tipped glass pipettes were prepared from unfilamented thin walled borosilicate glass tubing (Harvard apparatus, Kent, UK). Pipettes were pulled to a tip diameter of 4-5 μM using a Sutter P-87 puller (Sutter Instruments Co, Foster City, CA, USA) and fire polished using a Narishige microforge (Narishige Scientific Instrument Lab, Tokyo, Japan) to ensure a smooth tip. Patch pipettes were used within 8 hours of construction to ensure dust particles did not adhere to the glass and prevent seal formation between the pipette and the cell membrane. The pipettes were stored in a sealed electrode container (World Precision Instruments, Florida, USA) to prevent access of dust particles.

Patch pipettes were back filled using suction before being placed in an electrode holder connected to a manipulator-mounted headstage (Axon Instruments, Foster City, CA, USA). The headstage was connected to an Axoclamp 2B amplifier (Axon Instruments). The tip of the patch pipette was placed on the surface of a cardiac myocyte using a Narishige hydraulic micromanipulator (Narishige Co. Ltd, Tokyo, Japan). An offset of 2-4 mV was

typically observed due to the development of a liquid potential. This offset was not corrected, as it was not large enough to interfere with subsequent measurements.

Gentle suction was applied to the membrane directly under the membrane tip using the sidearm of the pipette holder. The cell was then lifted from the bottom of the tissue bath. A 20 mV, 40 ms depolarising square wave pulse was passed from the voltage clamp amplifier through the pipette at an interval of 1500 ms. The process of seal formation was monitored by observing the current elicited during the plateau of the sealing pulse. Once a seal had formed, a small amount of suction was occasionally applied to rupture the membrane. Rupture of the membrane allowed low resistance access to the interior of the cell through the pipette tip and perfusion of the intracellular compartment with the contents of the patch pipette. This state is termed the “whole cell configuration”. A video microscopy camera (CCD-72S series, Dage-MTI Inc, Michigan City, IN, USA) and video monitor (Matsushita Electric Industrial Co Ltd, Osaka, Japan) connected to the microscope allowed continuous visual monitoring of the cell during the experiments.

2.3.3. Experimental Solutions.

The myocytes suspended in the tissue bath were initially superfused with modified Tyrodes solution containing (in mM/L) 140 NaCl, 5.6 KCl, 2.16 CaCl₂, 0.44 NaH₂PO₄, 10 glucose, 1.0 MgCl₂, and 10 N-2-hydroxyethylpiperazine-N-2-

ethanesulphonic acid (HEPES). The solution also contained gentamicin 0.4 ml,(16mg/L) and was titrated to a pH of 7.4 at 35 °C with NaOH. After the whole cell configuration was established and membrane capacitance measured, the superfusates was switched to a modified Tyrodes solution similar to the one used above except that it was nominally Ca^{2+} free and contained 0.2 mM CdCl_2 . Cd^{2+} was included to block Ca^{2+} channel conductance and inhibit Na^+ - Ca^{2+} exchange current (Nakao and Gadsby, 1989). The solution also contained 2 mM BaCl_2 to inhibit conductance through K^+ channels (Tourneur et al., 1987). The composition of the pipette solution and the extracellular solutions were essentially designed to block any 'non pump' currents, leaving the Na^+ - K^+ pump current as the dominant remaining current. This facilitates its measurement. In some experiments the superfusate used was Na^+ free (See section 3.2 and 5.2). The Na^+ -free modified Tyrodes superfusate contained (in mM/L) 140 NMG (D(-)-methyglucamine), 0.44 NaH_2PO_4 , 10 glucose, 10 HEPES, 1 MgCl_2 , 0.2 CdCl_2 , 2 BaCl_2 , and 5.6 KCl. The solution also contained gentamicin 0.4 ml.. This solution is highly alkaline with a pH of ~10.4. It was titrated to a pH of 7.4 at 35 °C with concentrated HCl.

2.3.4. Pipette filling solutions.

The pipettes were back-filled using suction with a solution containing (in mM) 10 Na glutamate, 1 KH_2PO_4 , 5 HEPES, 2 Mg-ATP, 5 ethyleneglycolol-bis-(β -aminoethlyether)N,N,N',N-tetraacetic acid (EGTA), 70 K^+ -glutamate and 80 TMA-Cl. The solution was titrated to pH of 7.2 at 22°C with KOH. This was the

standard pipette solution unless otherwise stated. In experiments designed to examine effects of interventions on near-maximal pump turnover, the Na^+ concentration in pipette solutions was increased to 80 mM. The composition was similar to the solution described above except it contained 80 rather than 10 mM Na-glutamate; the concentration of TMA-Cl was reduced 10 mM to maintain the same osmolality of pipette solutions and superfusates. Pipette solutions were filtered through a 0.1 μM syringe filter (Pall Corporation, Ann Arbor, MI, USA). When filled with solutions, patch pipettes had resistances of 0.8-1.1 $\text{M}\Omega$. Peptide and non-peptide blocking agents of messenger molecules were included in patch pipette solutions when indicated. This allowed easy access of the blocking agents to the intracellular compartment and hence blockade of relevant messenger cascades. Details are described in the sections related to the experiments in which the blocking agents were used.

2.3.5. Measurement of membrane capacitance and access resistance.

Membrane capacitance (C_m) of the cell was estimated after whole cell configuration was established using the membrane test mode of Clampex 7 software. All currents measured were normalised using cell membrane capacitance and hence cell size as the C_m is indicative of the surface area. The membrane test mode also allowed estimation of the access resistance (R_a) which reflects the quality of access between the patch pipette and the

intracellular compartment. The experiment was abandoned if R_a was greater than $\sim 4 \text{ M}\Omega$.

2.3.6. Measurement of I_p

Once the R_a was found to be acceptable, myocytes were voltage clamped at a test potential known to inactivate voltage sensitive Na^+ channels (Follmer et al., 1987). The test potential was -40 mV unless indicated otherwise. A trace of the holding current (I_h — the current required to hold the membrane potential at the test potential) was recorded using Axotape version 2 software. Once I_h was stable the superfusates were switched to Ca^{2+} free, Cd^{2+} and Ba^{2+} containing Tyrodes solution to inhibit Na^+ - Ca^{2+} exchange and K^+ channel conductance (Tourneur et al., 1987). This causes an inward current shift of membrane current to a new steady state level. The cell was left in this solution for approximately 10 minutes, ensuring a stable plateau in current was reached for the final 30 seconds. The superfusate was then switched to a Ca^{2+} -free, Ba^{2+} containing solution containing $100 \mu\text{M}$ ouabain (Sigma Chemical Co., St Louis, MO, USA) to block the electrogenic Na^+ - K^+ pump current. This concentration of ouabain results in complete inhibition of the Na^+ - K^+ pump (Hool et al., 1995a). The pump current (I_p) generated by the Na^+ - K^+ pump is identified by an inward shift in membrane current induced by ouabain. I_p was identified at a holding potential of -40 mV as the difference between stable plateaux of holding current before and after Na^+ - K^+ pump blockade with $100 \mu\text{mol/L}$ ouabain. It is crucial for validity of data that I_h is “stable” before ouabain is superfused. “Stable”

holding currents were defined according to criteria published previously (William et al., 2005a). A stable current was identified when no drift could be identified on the digital display of the voltage clamp amplifier for at least 50 s. The plateaus were defined by the means of 5 samples of the holding current obtained with an electronic cursor taken at ~5 sec intervals before and after exposure to ouabain. Sampling rate for all recordings was 1 per sec. An example of holding currents recorded during an experiment is shown in Figure 2.2. This shift was measured using an electronic cursor according to a protocol described previously (William et al., 2005a)

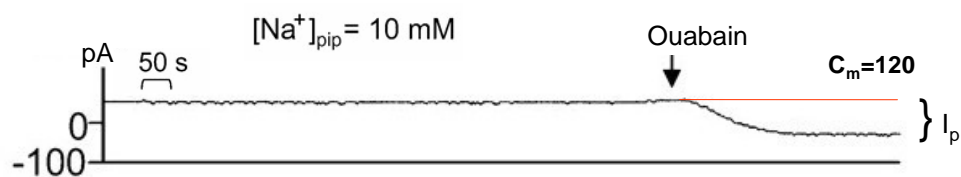


Figure 2.2 Trace of ouabain induced shift in holding current

Electrical trace of one cardiac myocyte using the patch clamping technique. The arrow indicates the addition of ouabain to the perfusing solution and subsequent change in current measured. I_p was identified as the ouabain induced shift in the holding current normalised for membrane capacitance (C_m). Na^+_{pip} = the concentration of Na^+ in mM in the patch pipette

2.4. Data acquisition, analysis and storage.

Recording of membrane current, holding potential, passing of current and voltage steps were performed using continuous single electrode voltage-clamp mode of an Axoclamp 2B amplifier via DigiData 1200 series interface (Axon Instruments, Foster City, CA, USA). Voltage clamp pulses and stimulus protocols were applied using the Clampex 7 software run on an IBM PC300 GL computer. Continuous recording of V_m and I_h were made using a multichannel computerised data acquisition system with 12 bit resolution (Axotape Version 2, Axon Instruments). Signals were displayed on an IBM 6546-01S monitor.

2.5 Chemicals and Reagents

The following chemicals were sourced from Sigma Chemical Co. (St Louis, Missouri, USA); potassium hydroxide (KOH), sodium hydroxide (NaOH), potassium chloride (KCl), ouabain, cadmium chloride (CdCl_2), adenosine 5-triphosphate magnesium salt (Mg.ATP), ethyleneglycol-bis-(β -aminoethylether)N,N,N',N-tetraacetic acid (EGTA) ($\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_{10}$), sodium chloride (NaCl), calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$), magnesium sulphate (MgSO_4), taurine ($\text{C}_2\text{H}_7\text{NO}_3\text{S}$), potassium L-glutamate ($\text{C}_5\text{H}_8\text{KNO}_4 \cdot \text{H}_2\text{O}$), monosodium glutamate ($\text{C}_5\text{H}_8\text{NNaO}_4 \cdot x\text{H}_2\text{O}$).

Tetramethylammonium chloride (TMA,Cl) was sourced from Fluka Chemicals (Switzerland).

The following chemicals were sourced from Merck (Darmstedt, Germany); sodium hydrogen carbonate (NaHCO_3), NMG (D(-)-methlyglucamin($\text{C}_7\text{H}_{17}\text{NO}_5$), hydrochloric acid (HCl).

Gentamicin was sourced from Pfizer (Brooklyn, New York, USA).

The following reagents were sourced from BDH (Australia); potassium dihydrogen orthophosphate (KH_2PO_4), glucose anhydrous ($\text{C}_6\text{H}_{12}\text{O}_6$), sodium dihydrogen orthophosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), barium chloride (BaCl_2).

2.6 Statistical analysis

Results are expressed as mean \pm standard error of the mean (SEM). When comparing two groups we used a Students t-test and a Welch corrected if the data was found to have significantly different standard deviations. A one way ANOVA was used to compare multiple groups with a Dunnett's post hoc test. Graphpad Instat Ver. 3.0. was used for calculations. A P value of <0.05 is regarded as significant in all comparisons.

Chapter 3

Regulation of the cardiac $\text{Na}^+\text{-K}^+$ pump by NOS- derived nitric oxide

3.1. Introduction.

Exogenous NO supplied by a pharmacological donor compound stimulates the $\text{Na}^+\text{-K}^+$ pump in isolated cardiac myocytes (William et al., 2005a). In the intact cell, NO is synthesised by NOS, whose major substrate is L-arginine (L-arg) as shown in Figure 1.6. Since NOS is expressed in cardiac myocytes, endogenously synthesised NO may be an important determinant of sarcolemmal $\text{Na}^+\text{-K}^+$ pump activity. However, this cannot be assumed: effects of NO can be highly compartmentalized in molecular microdomains (Hess et al., 2005) and results obtained using a pharmacological NO donor may be critically different from effects of endogenously synthesised NO. In this Chapter, the theory that NO is produced endogenously from L-arg by NOS is examined. For the hypothesis to be examined is it important that NO is metabolised to biologically active compounds with properties distinctly different from the properties of NO, in particular the peroxynitrite derivative of NO, ONOO^- (Figure 1.8).

The K_m (the Michealis constant- substrate concentration at which reaction is half V_{\max}) of L-arg for the different isoforms for NOS are reported to range from 2.9 μM to 30 μM (Sydow and Munzel, 2003, Closs et al., 2000). The concentration of L-arg found within the cell is usually in the range of 1-2 mM, a concentration

far above the low K_m of NOS. However, *in vitro* studies have shown that the addition of L-arg to the intracellular compartment stimulates an increase in NO production (Wang et al., 2004). This is known as the L-arg paradox (Kurz and Harrison, 1997). It is important to examine the effect of NO produced endogenously in cardiac myocytes on the $\text{Na}^+\text{-K}^+$ pump. Experiments were performed to examine if inclusion of L-arg in the patch pipette solution, used to perfuse the intracellular compartment, could alter the pump current of the cardiac $\text{Na}^+\text{-K}^+$ pump. Experiments were later performed to examine if this increased activity was due to a response to increased NO production, stimulated by the increase in the substrate L-arg. The downstream messenger of NO, soluble guanylyl cyclase (sGC), was inhibited to examine this. We also examined if the increase in I_p could be altered by some other effect such as an alteration in the NOS co-factors. BH_4 is a co-factor of NOS. It is required in the redox reaction and donates an electron to a haem-dioxy intermediate during the NOS oxygen cascade. A reduction in the concentration of BH_4 can lead to uncoupling of NOS and the production of reactive oxygen species such as peroxynitrate and superoxide (Vasquez-Vivar et al., 2002)(see Figure 1.5). We examined the effect of the addition of BH_4 to the patch pipette solution on I_p .

3.2. Methods.

Cardiac myocytes were patch clamped using pipettes that included 10 mM Na^+ . The solutions were free of L-arg or they contained 1 μM , 10 μM , 100 μM or 1mM L-arg. A standard Na^+ -containing superfusate was used in the initial

series of experiments. Details of cardiac myocyte isolation and whole cell patch clamp technique are discussed in sections 2.2 and 2.3. Details of the composition of pipette and superfusate solutions are given in sections 2.3.4 and 2.3.3.

In principle, perfusion of the intracellular compartment with pipette solution might deplete the NOS co-factor tetrahydrobiopterin (BH₄) and have an effect on baseline pump currents. To examine if supplementation of BH₄ had an effect on I_p we included it in solutions in a concentration of 10 μM.

Whenever an experimental intervention induces an increase in I_p, one must consider if this increase might be due to a transmembrane influx of Na⁺ into the myocyte from the extracellular compartment. If such an influx is large, the intracellular Na⁺ concentration cannot necessarily be expected to be controlled by the perfusion of patch pipette solution through the intracellular compartment. The possibility of transmembrane Na⁺ influx can be eliminated when a Na⁺-free superfusate is used. Additional experiments were performed using such a Na⁺-free superfusate. Osmotic balance was maintained by the addition of D(-)-N-Methylglucamine (NMG) to the superfusate. Details of its composition are given in Section 2.3.3. Patch pipette solutions included 10 μM L-arg. Later experiments to examine Na⁺-K⁺ pump activity and turnover used a pipette solution Na⁺ concentration of 80 mM. This is indicated in the text where used.

L-NAME (N(G)-nitro-L-arginine methyl ester) (SIGMA) was used to inhibit NOS. L-NAME is an analogue competitive inhibitor of L-arg. A concentration of 10 μM was used. L-NAME was dissolved in H₂O.

A specific inhibitor of NO-activated soluble guanylyl cyclase (sGC), 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ),(Calbiochem LaJolla, Ca) was used to examine the downstream messengers of NO. Pipette solutions included 10 μ M ODQ and 10 μ M L-arg. ODQ was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in solution was 0.037%. DMSO in this concentration has no effect on I_p .

3.3. Results.

The ouabain-induced shift in holding current, normalised for membrane capacitance was larger for the myocyte perfused intracellularly with the pipette solution containing L-arg than for the control myocytes (Figure 3.1). Figure 3.2 shows the mean I_p s measured using the different L-arg concentrations. At 10 μ M L-arg, maximal stimulation of I_p is reached. Thus, this concentration of L-arg was used in the pipette solution for all further experiments unless stated.

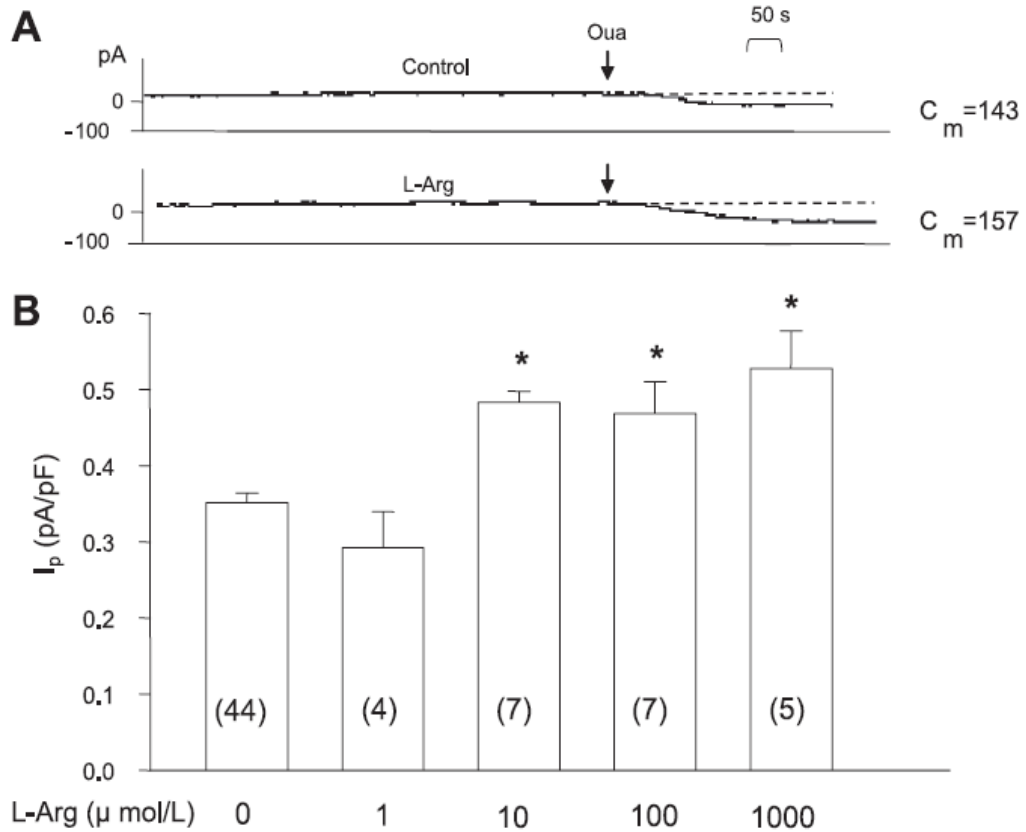


Figure 3.1 Effect of L-arginine on Na^+K^+ pump current in myocytes

A trace of a holding current of a ventricular myocyte isolated from a rabbit and voltage clamped at -40 mV. $[\text{Na}^+]_{\text{pip}}$ was 10 mM and $[\text{K}^+]_{\text{pip}}$ was 70 mM. The timing of exposure to Ba^{2+} and ouabain is indicated. While the time of onset and exposure *in vitro* varied between sets of experiments, I_p was determined after a fixed time interval within each experiment. I_p was defined as the ouabain-sensitive shift in the holding current, measured with an electronic cursor. Panel A is a representative trace of a control series, while panel B represents the holding current measured when L-arg is added to the pipette solution.

The increase in I_p induced by L-Arg could be due to an intrinsic increase in Na^+ - K^+ pump activity or it could be, indirectly, due to enhanced influx of Na^+ into the myocytes and secondary pump stimulation. To distinguish between direct and indirect pump stimulation we used Na^+ free superfusate solutions. The possibility of transmembrane Na^+ influx is eliminated when a Na^+ -free superfusate is used. Details of its composition are given in Section 2.3.3. Osmotic balance was maintained by the addition of NMG to the superfusate. Mean I_p of myocytes perfused or not perfused with L-arg and studied in Na^+ -free superfusates are presented in Figure 3.3. In agreement with a previous study, (William et al., 2005a) mean I_p measured with patch pipettes that did not contain L-arg was lower in Na^+ -free than Na^+ -containing superfusates (compare Figures 3.2 and 3.3). However, the L-arg induced stimulation of I_p persisted when Na^+ influx was eliminated by the use of Na^+ -free extracellular solutions.

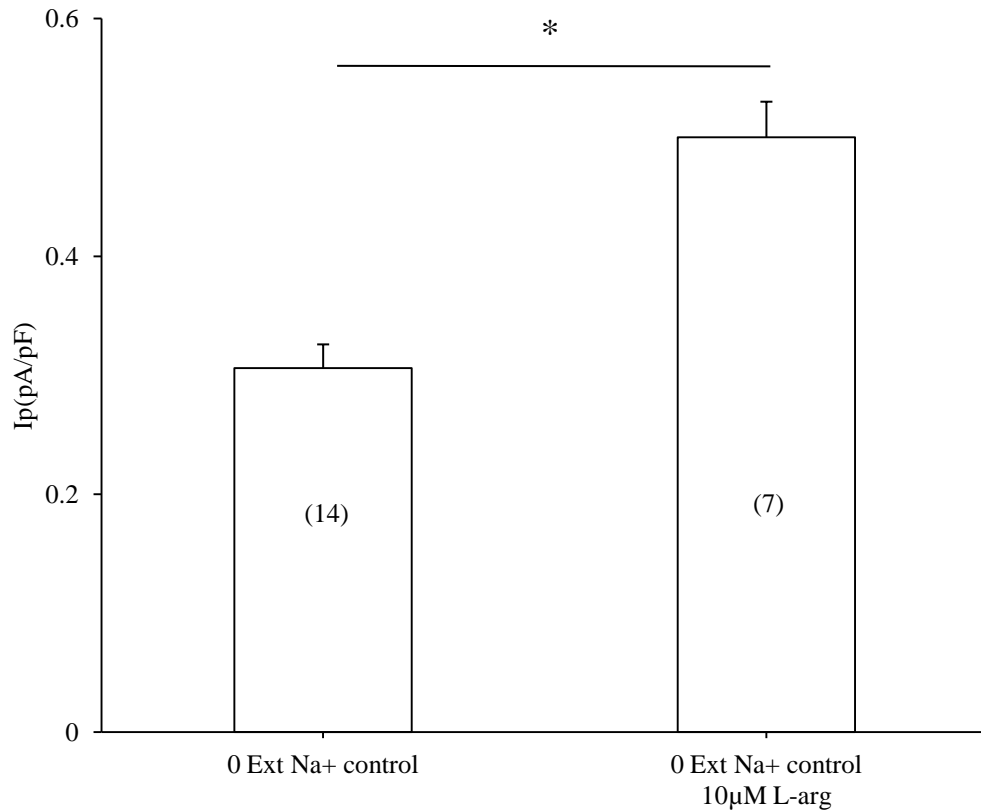


Figure 3.2 Effect of L-arginine on I_p with 0 External Na⁺

Myocytes were patched clamped with Na⁺-free extracellular superfusate and L-arg – free and L-arg (10 µM) containing pipette solutions. L-arg induced a highly significant (* $p < 0.0001$) stimulation of I_p . As there was no Na⁺ externally this effect can not be attributed to a transmembrane influx of Na⁺. The numbers in parentheses indicate the number of experiments in each group.

To examine if L-Arg enhances maximal $\text{Na}^+\text{-K}^+$ pump turnover we used patch pipette solutions that included 80 mM Na^+ (osmotic balance was maintained by adjusting the concentration of TMA.Cl). The solutions also included L-arg or they were L-arg-free. A Na^+ containing superfusate was used. These results are shown in Figure 3.4. L-arg did not induce an I_p higher than the control, indicating that L-arg does not have an effect on pump turnover.

Figure 3.1 shows examples of membrane holding currents during measurements of I_p of a myocyte perfused with pipette solution that was free of L-arg and a myocyte perfused with pipette solutions containing 10 μM L-arg.

It is important to ascertain that the L-arg induced $\text{Na}^+\text{-K}^+$ pump stimulation is due to enhanced synthesis of NO. In principle, this might be achieved by examining the effect of inhibiting NOS with N(G)-nitro-L-arginine methyl ester (L-NAME). However, since L-NAME is a relatively weak competitive inhibitor of L-arg, it is not expected to be effective at a high concentration of L-arg. As an alternative option, the downstream effects of NO were inhibited by including a specific inhibitor of NO-activated guanylyl cyclase, 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ), in patch pipette solutions, thus linking NO to stimulation of the $\text{Na}^+\text{-K}^+$ pump. Pipette solutions included 10 μM ODQ and 10 μM L-arg. Control experiments were also performed using solutions containing ODQ but no L-arg. The superfusate contained Na^+ . Results are shown in Figure 3.5. ODQ abolished the L-arg induced $\text{Na}^+\text{-K}^+$ pump stimulation.

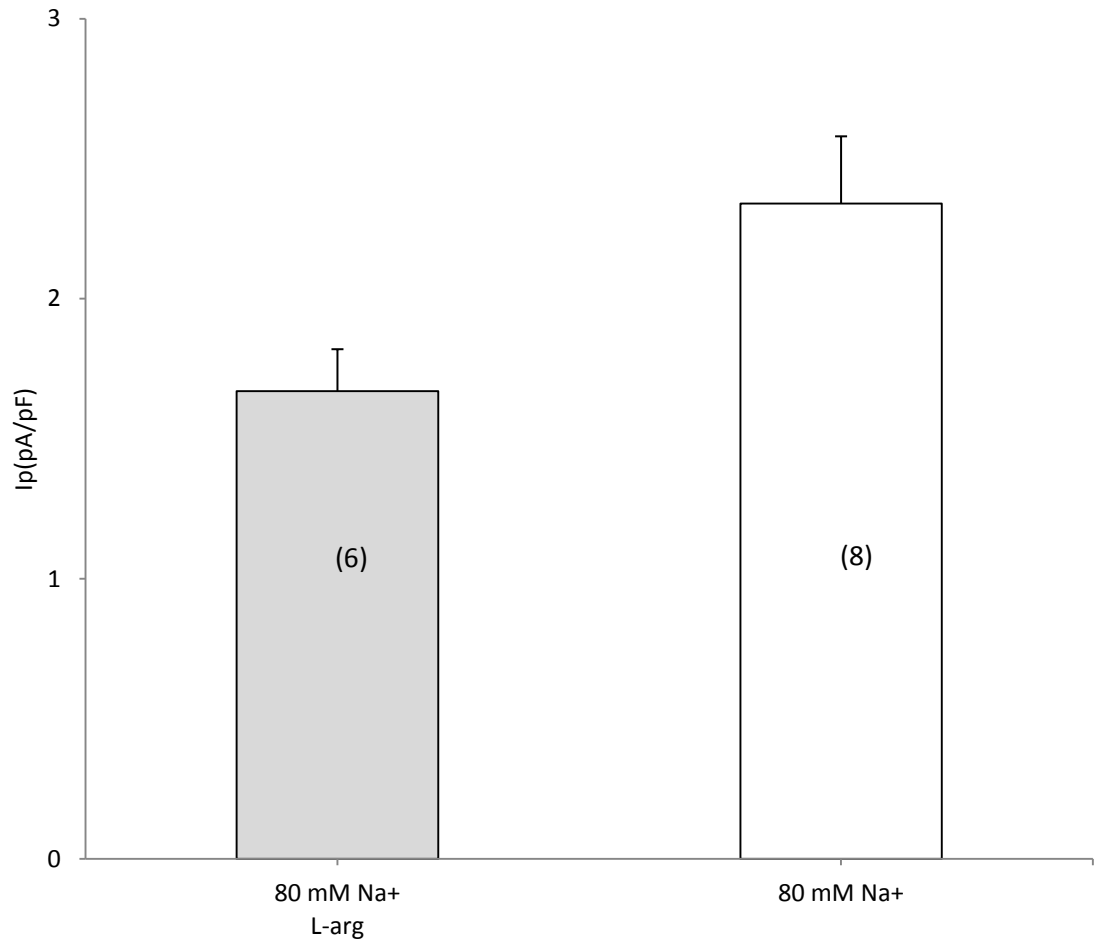


Figure 3.4. The effect of 80 mM Na⁺ on I_p .

Myocytes were patch clamped with 80 mM Na⁺ in the patch pipette solutions. The pipette solutions contained L-arg or were L-arg free. The addition of L-arg to the pipette solution did not increase I_p . This demonstrates that L-arg did not affect maximal pump turnover. The numbers in parentheses indicate the number of experiments in each group.

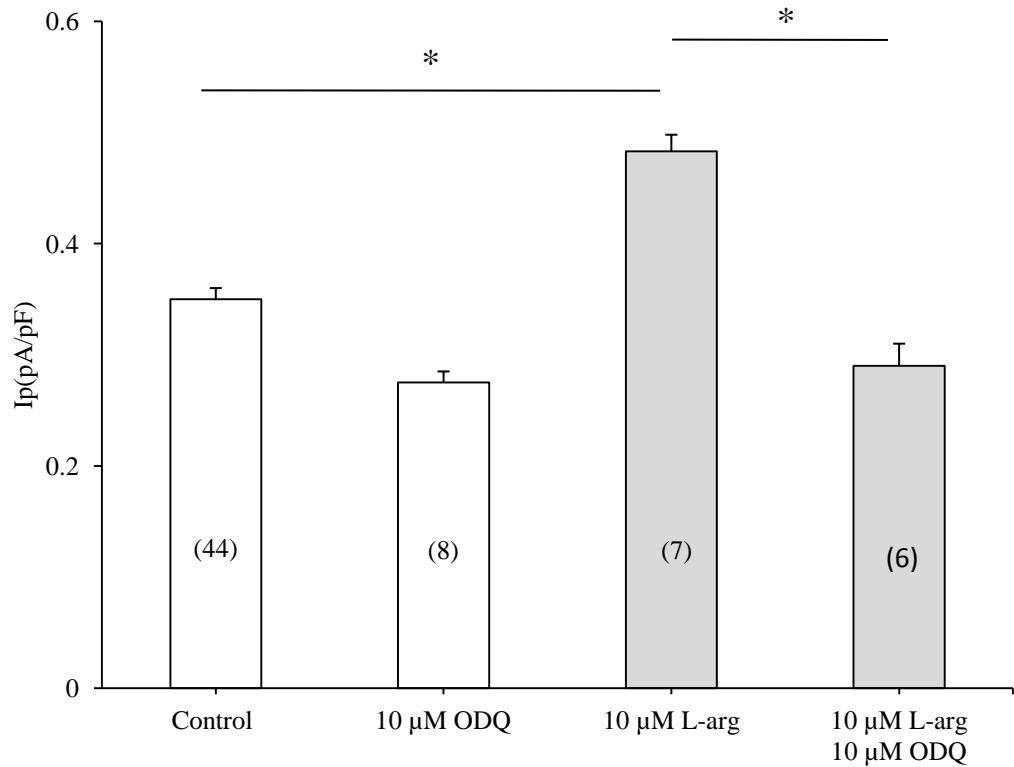


Figure 3.5 The effect of ODQ on L-arg induced Na^+ - K^+ pump stimulation

Myocytes were patch clamped with pipette filling solution containing ODQ (sGC inhibitor), L-arg or a combination of both. There was a no difference in I_p between controls and ODQ alone in the pipette solution. There is a highly significant increase in I_p with the addition of L-arg to the pipette solution ($*p < 0.001$). This stimulation of I_p is abolished with the addition of ODQ to the pipette solution ($*p < 0.001$). The numbers in parentheses indicate the number of experiments in each group. The pipette solution contained 10 μM Na^+ .

In theory, perfusion of the intracellular compartment with pipette solution might deplete the NOS co-factor, tetrahydrobiopterin (BH₄), and have an effect on baseline pump currents. To examine if supplementation of BH₄ had an effect on I_p we included it in the pipette solution at a concentration of 10 μM. The solutions did not contain L-arg. Mean I_p (0.32 ± 0.03 pA/pF, N = 5) was not significantly different to mean I_p of control myocytes (0.35 ± 0.01 pA/pF, N = 44). BH₄ was also included in the pipette solution with L-arg. There was no significant difference observed (See Figure 3.6).

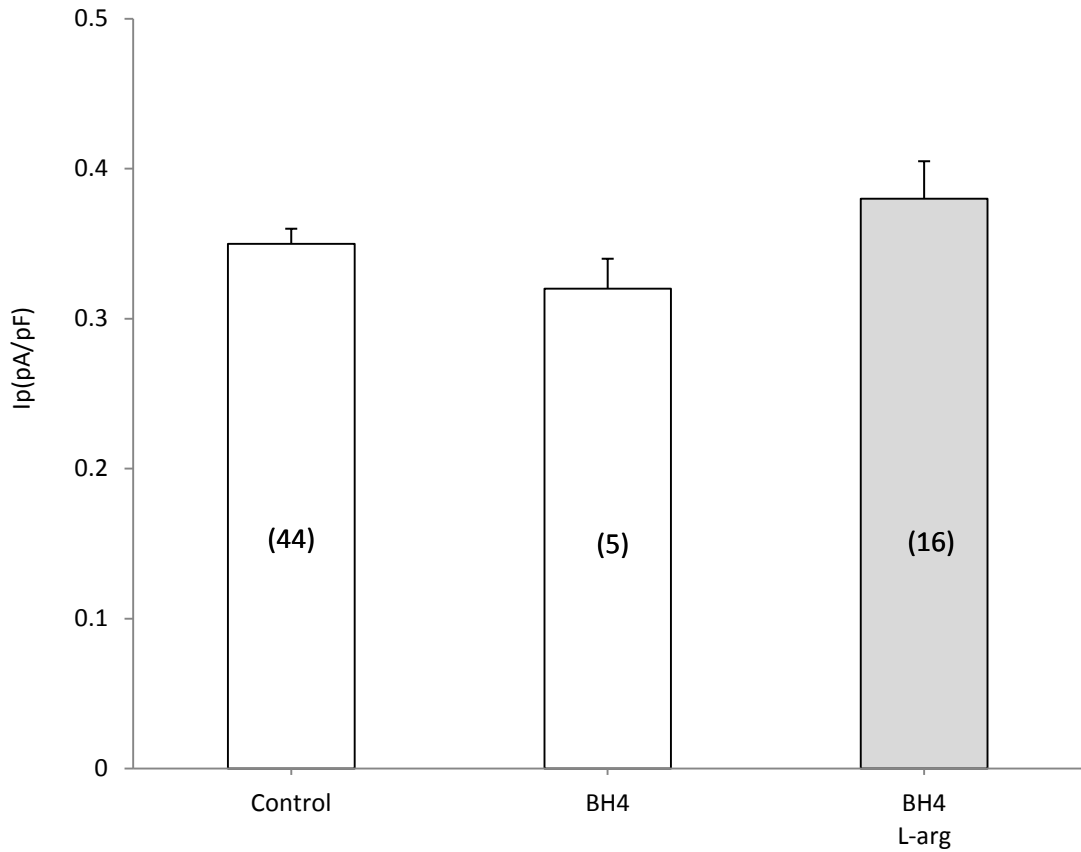


Figure 3.6 Effect of BH₄ on control myocytes and effect of BH₄ on L-arg induced Na⁺-K⁺ pump stimulation

Myocytes were patch clamped with pipette filling solution containing BH₄. There was no significant difference in I_p between control myocytes and those containing BH₄ or those containing both BH₄ and L-arg.

The numbers in parentheses indicate the number of experiments in each group. The pipette solution contained 10 mM Na⁺.

3.4. Discussion.

The results shown in Figure 3.2 indicate that supplementing the patch pipette solution with L-arg induces an increase in mean I_p . There was no increase in I_p when the L-arg concentration was 1 μ M, a value well below K_m for the various isoforms of NOS (Closs et al., 2000, Vukosavljevic et al., 2006). However, the L-arg-induced Na^+ - K^+ pump stimulation seemed to saturate at concentrations of 10 μ M. The cytosolic L-arg concentration, at least in endothelial cells, is reported to range from 0.1-2.0 mM, *i.e.* concentrations expected to fully saturate NOS (Vukosavljevic et al., 2006, Closs et al., 2000). The simplest interpretation of the data shown in Figure 3.2 is that perfusion of the intracellular compartment with patch pipette solutions not containing L-arg depletes the cell of L-arg to a level where its concentration becomes rate limiting for NOS-mediated NO synthesis. This suggests that the pump current measured *in vitro* using “control” pipette solutions free of L-arg may not fully reflect the *in vivo* pump activity where there is an abundance of endogenously produced NO and its downstream messengers.

There is some evidence to suggest that separate cellular pools of L-arg exist that are not freely exchangeable, at least in endothelial cells (Closs et al., 2000, Vukosavljevic et al., 2006). From a functional perspective, L-arg contained in caveolae appears separate from L-arg in the bulk phase of the cytosol, and co-localisation of L-arg transporters and eNOS may be part of a functional unit (Mann et al., 2003, Vukosavljevic et al., 2006). This may explain the apparent “L-arg paradox”: the finding that extracellular supplementation of L-arg in

modest concentrations increases cellular NO synthesis despite the intracellular concentration being at a level expected to saturate all NOS isoforms.

If a separate L-arg pool in membrane caveolae exists in cardiac myocytes, it should not be assumed that the concentration of L-arg in pipette solutions reflects the concentration at the site of NO synthesis and its downstream effects on the Na⁺-K⁺ pump in this study. The L-arg induced Na⁺-K⁺ pump stimulation could be due to a direct effect of NO on the pump molecule or the lipid membrane they are embedded in. NO is highly soluble in lipids and increases membrane fluidity, (Goligorsky et al., 2001, Goligorsky and Gross, 2001), a known determinant of Na⁺-K⁺ pump activity (Cornelius et al., 2001). NO can also alter protein function by S-nitrosylation of reactive cysteine residues (Hess et al., 2005). However, the effect of ODQ to abolish the increase in I_p induced by L-arg (Figure 3.5) strongly implicates downstream activation of sGC, rendering a direct effect of NO unlikely. It has been previously found that activation of sGC using a pharmacological NO donor or YC-1 stimulates the Na⁺-K⁺ pump in cardiac myocytes (William et al., 2005a) via downstream messengers that include cGMP-activated protein kinase (PKG). While kinase-mediated phosphorylation has been implicated in Na⁺-K⁺ pump stimulation, phosphorylation sites on the pump molecule are poorly accessible (Sweadner and Feschenko, 2001) and the role of phosphorylation in pump regulation is uncertain (Cornelius et al., 2001). Phosphorylation of phospholemman, a membrane protein closely associated with the Na⁺-K⁺ pump has been suggested to regulate the pump (Sweadner, 2005). However, phosphorylation of phospholemman is unlikely to mediate NO-dependent pump stimulation because the protein is not phosphorylated by PKG (Sarcevic et al., 1989)

Regardless of uncertainties regarding intracellular compartments for L-arg the experiments presented in this section show that endogenously synthesised NO can stimulate the sarcolemmal $\text{Na}^+\text{-K}^+$ pump. This has not been shown previously.

Chapter 4

Uncoupling of Nitric Oxide Synthase

4.1 Paraquat induced uncoupling of NOS

Results presented in Chapter 3 (Section 3.2) showed that endogenously synthesised NO stimulates Na⁺-K⁺ pump function in isolated cardiac myocytes. This is in agreement with the previously demonstrated effect of exogenous NO supplied by pharmacological donors (William et al., 2005a). However, metabolic pathways of NO synthesis are closely linked to pathways generating O₂⁻ and ONOO⁻ and these ROS/RNS may have inhibitory effects on the pump as referred to in Section 1.5. Differential regulation of NO vs. ROS/NOS synthesis might offer an effective mechanism for increasing or decreasing Na⁺-K⁺ pump activity. Experiments were designed to examine if endogenous production of ROS/NOS regulates the Na⁺-K⁺ pump. The bipyridyl herbicide paraquat was used for this purpose.

When paraquat is added to myocytes, NOS can become “uncoupled” and preferentially synthesize O₂⁻, which often has opposing effects to NO (Margolis et al., 2000). The mechanism for paraquat-induced uncoupling is illustrated in Figure 4.1.

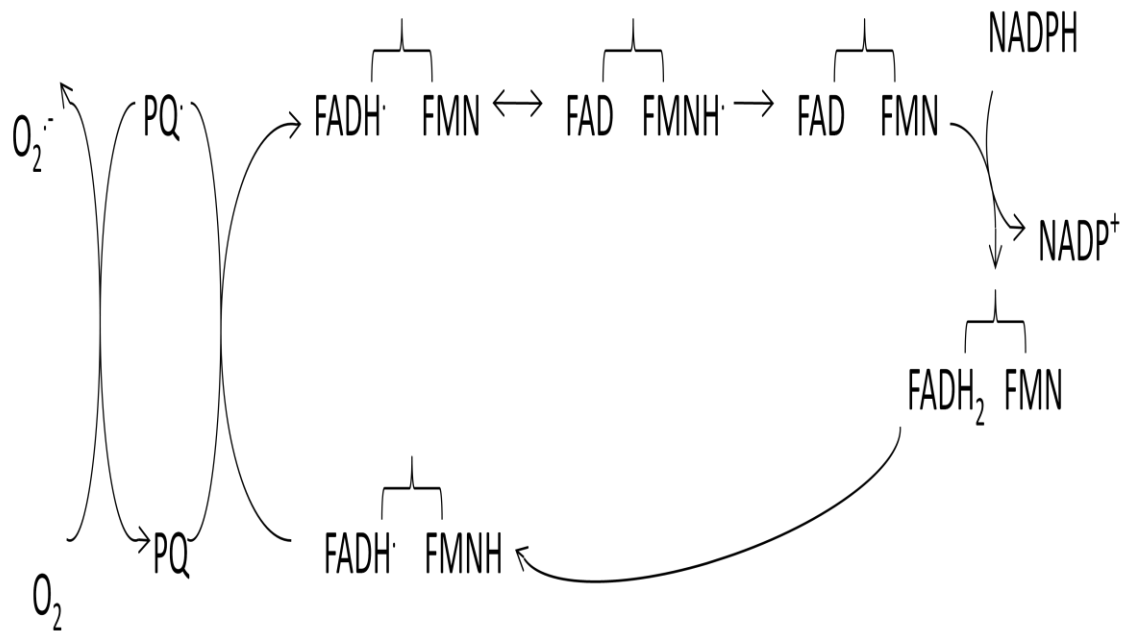


Figure 4.1 Electron flow in the reductase domain of NOS I in the presence of paraquat

Electrons from NADPH are transferred to FAD, resulting in formation of FADH₂ which, upon disproportionation with the flavin mononucleotide (FMN), leads to synthesis of the flavin free radicals FADH· and FMNH·. When NOS operates in the coupled mode, FMNH· transfers an electron to Fe³⁺ molecule of the oxidase domain. With the addition of paraquat, electrons are shunted away from the heme to form paraquat free radical (PQ·). An electron is transferred from PQ· to molecular O₂, and O₂^{·-} is generated. (FMH- flavin mononucleotide, FAD- flavin adenine dinucleotide, NADP- nicotinamide adenine dinucleotide phosphate). Adapted from Margolis *et al* 2000, *Biochim Biophys Acta* 1524, 253-257.

4.2. Methods

Patch pipette solutions contained 10 mM Na⁺ and a standard Na⁺-containing superfusate was used in all experiments (see sections 2.3.3 and 2.3.4 for details of composition). The pipette solutions contained 10 μM L-arg or they were free of L-arg. Paraquat (Sigma-Aldrich St Louis, Missouri, USA) in a concentration of 100 μM was dissolved directly in the pipette solution without the use of an additional solvent. Patch pipette solutions containing superoxide dismutase (SOD) (bovine erythrocyte) (Sigma-Aldrich St Louis ,Missouri, USA) in a concentration of 200 IU/mL (Zhang et al., 2002) were also used. SOD was dissolved directly in the pipette solution.

The ONOO⁻ scavenger ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) (Sigma-Aldrich pharmaceuticals, NSW, Au) was used at a concentration of 10 μM to examine the effect of eliminating the ROS/RNS produced by paraquat (Daiber et al., 2000). The final concentration of DMSO in the pipette solution was 0.1 %. DMSO in this concentration has no effect on I_p (data not shown). The control superfusate was used as described in section 2.3.3.

4.3. Results.

In an initial series of experiments the effect of paraquat in the absence of L-arg in pipette solutions was examined. Mean I_p of control myocytes and the I_p of myocytes patch clamped using paraquat-containing pipette solutions were compared. Results are included in Figure 4.2. There was not a significant difference between I_p with paraquat alone in the pipette solution. In a second

series of experiments, the effect of paraquat was examined with supplementation of the NOS substrate L-arg. L-arg was included in patch pipette solutions at a concentration of 10 μM . Pipette solutions also contained paraquat or they were paraquat-free. Results are included in Figure 4.2. Paraquat induced a large, statistically significant decrease in I_p when L-arg was supplemented. The effects of paraquat shown in Figure 4.2 are consistent with $\text{Na}^+\text{-K}^+$ pump inhibition induced by $\text{O}_2^{\cdot-}$. To obtain additional support for this hypothesis, experiments were designed to examine the effect of paraquat when $\text{O}_2^{\cdot-}$ was eliminated by the inclusion of SOD in patch pipette solutions. I_p of myocytes patch clamped using pipette solutions containing L-arg and paraquat only was compared with I_p of myocytes patch clamped using pipette solutions containing L-arg, paraquat and SOD. SOD eliminated the paraquat-induced decrease in I_p . Results are included in Figure 4.3.

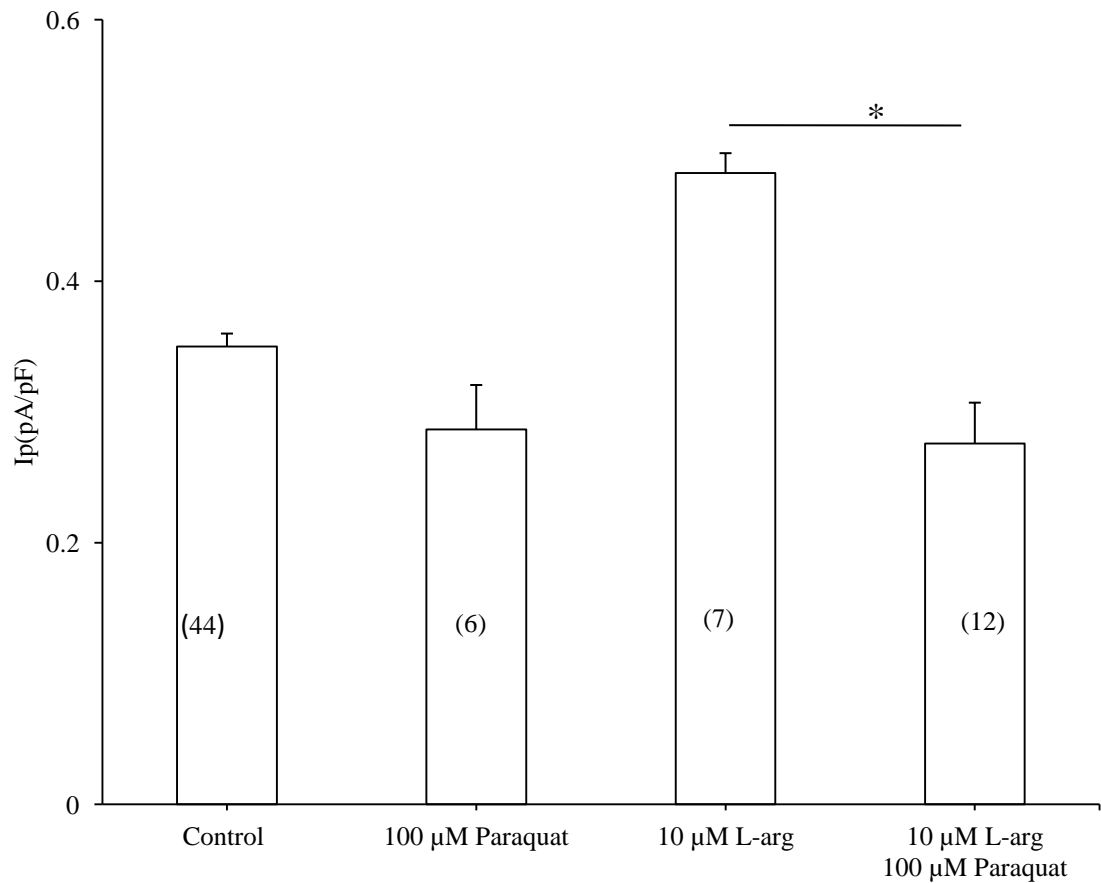


Figure 4.2 Effect of paraquat on L-arginine induced Na⁺-K⁺ pump stimulation

Myocytes were patch clamped with pipette solutions containing 100 μM paraquat alone, 100 μM paraquat and 10 μM L-arg or 100 μM paraquat, 10 μM L-arg and 200U/ml SOD. The addition of paraquat to the L-arg pipette solution caused significant inhibition of the Na⁺-K⁺ pump (*p<0.0001). Statistically significant difference is indicated by *. The numbers in parentheses indicate the number of experiments in each group.

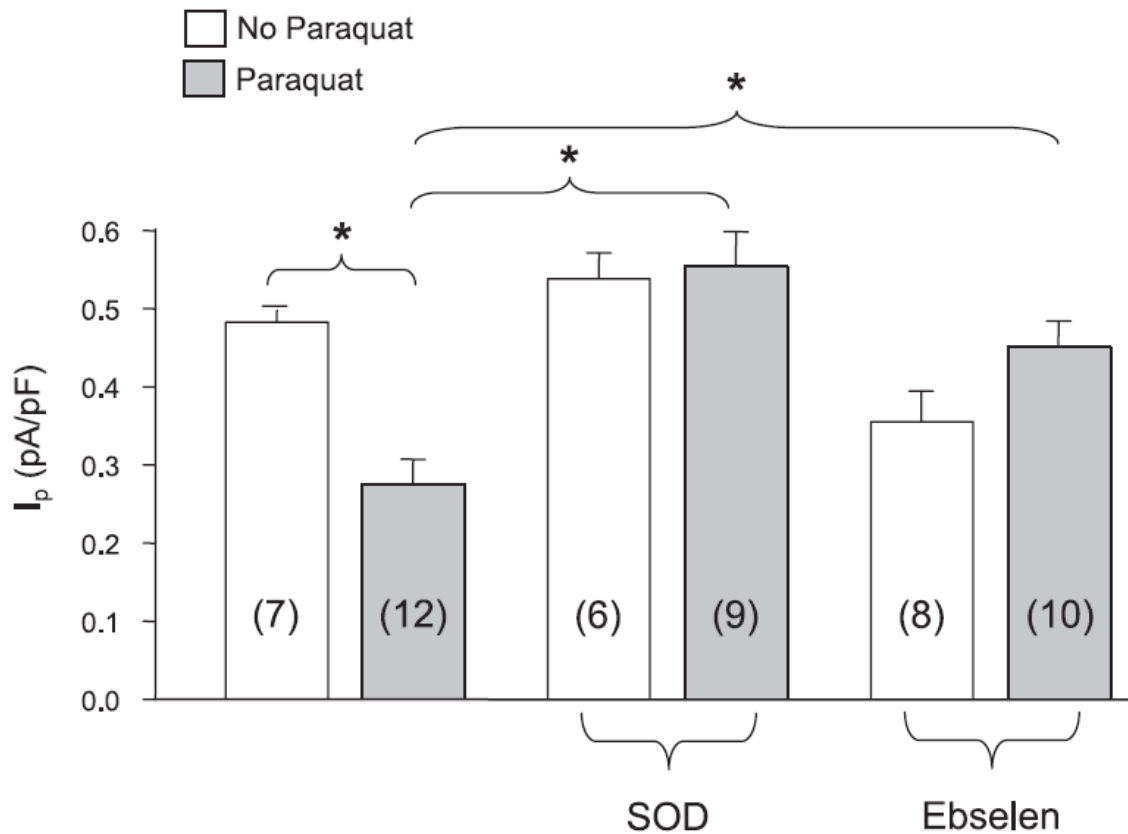


Figure 4.3 Effect of Superoxide Dismutase on paraquat induced $\text{Na}^+\text{-K}^+$ pump inhibition

Myocytes were patch clamped with pipette filling solution containing $10 \mu\text{M}$ L-arg and/or 200 IU/ml of SOD. The addition of L-arg to the pipette solution caused significant stimulation of I_p (data not shown). The addition of SOD to the pipette solution prevented the inhibition of the $\text{Na}^+\text{-K}^+$ pump, caused by oxygen radicals generated from the uncoupling of NOS by paraquat ($*p < 0.0001$) (as shown in Figure 4.2). Statistically significant differences are indicated by *. The numbers in parentheses indicate the number of experiments in each group.

We also examined the effect of the reactive oxidant scavenger ebselen. Ebselen was included in pipette solutions in a concentration of 10 μ M. Results are shown in Figure 4.4. Ebselen caused a decrease in I_p in the absence of paraquat in the pipette solution. This is consistent with a previously reported decrease in Na^+ - K^+ ATPase activity *in vitro* (Borges et al., 2005)(Borges et al., 2005)(Borges et al., 2005)(Borges et al., 2005) where higher concentrations of ebselen caused inhibition of Na^+ - K^+ ATPase by reacting with available cysteine residues.(Borges et al., 2005) In theory, if NOS is uncoupled and ebselen is scavenging the unstable oxygen radicals which are being produced, the cysteine molecule on the Na^+ - K^+ pump may escape modification. Inhibition of the Na^+ - K^+ pump is thus prevented, despite the uncoupling of NOS, as seen in the following series of experiments. Ebselen caused an increase in I_p in the presence of paraquat, implying a role for ROS/RNS in the inhibition of the Na^+ - K^+ pump.

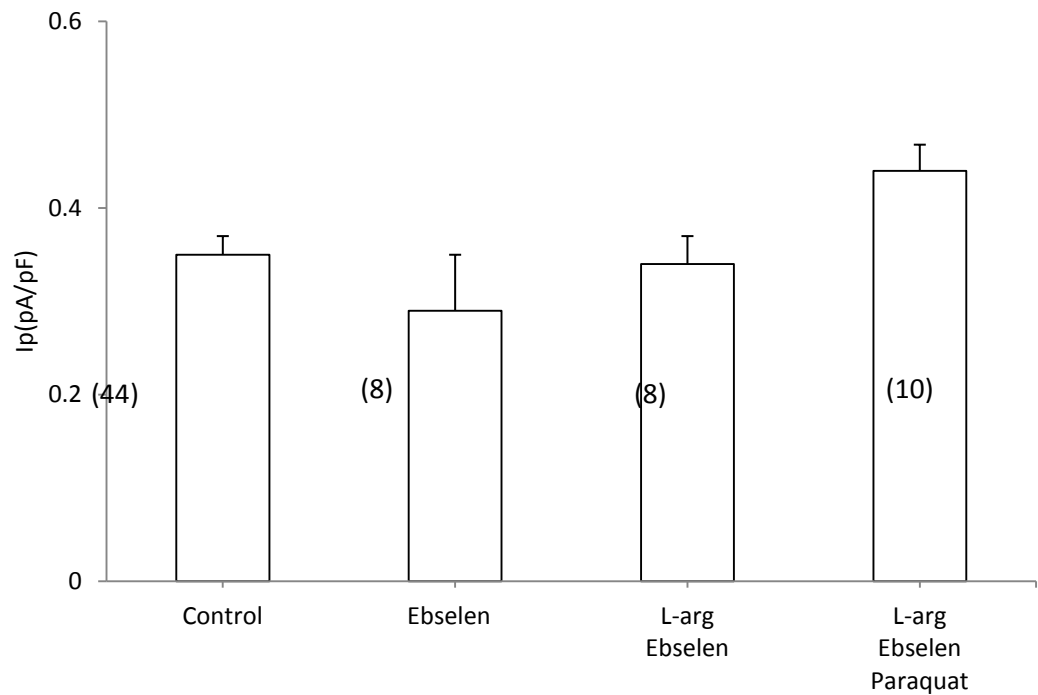


Figure 4.4 Effect of superoxide scavenger Ebselen on paraquat induced $\text{Na}^+\text{-K}^+$ pump inhibition

Myocytes were patch clamped with pipette filling solutions containing $10 \mu\text{M}$ L-arg and either $10 \mu\text{M}$ ebselen or $100 \mu\text{M}$ paraquat or both. The addition of ebselen to the pipette solution containing paraquat abolished the inhibition induced by paraquat (see Figure 4.2). Thus implicating the production of ROS/RNS as a significant inhibitor of I_p . The numbers in parentheses indicate the number of experiments in each group.

4.4.Discussion.

The purpose of the experiments in this Chapter was to establish if the inhibitory effect of ROS/RNS on the Na⁺-K⁺ pump, can be reproduced when an endogenous source of O₂⁻ is activated in an intact cell. The large decrease in I_p induced by paraquat when pipette solutions contained L-arg suggests that NOS activity is part of the mechanism for the inhibition. This, in turn, is consistent with Na⁺-K⁺ pump inhibition induced by uncoupled NOS-mediated synthesis of O₂⁻.

Since L-arg can directly scavenge O₂⁻ (Lass et al., 2002) it may have eliminated or reduced levels of O₂⁻ and hence relieved inhibition of the Na⁺-K⁺ pump induced by O₂⁻. However, the IC₅₀ of L-arg for scavenging of O₂⁻ is ~75 μM (Lass et al., 2002) while the effect of L-arg on I_p found in this study was saturated at a concentration of 10 μM, therefore the concentration of L-arg in the pipette solution is not high enough to have a direct scavenging effect. In addition, there was no significant effect of SOD in pipette solutions unless NOS was uncoupled with paraquat (Figure 4.3) suggesting that elimination of constitutively synthesized O₂⁻ does not account for the increase in I_p when the intracellular compartment is supplemented with L-arg.

SOD eliminates O₂⁻ in a highly specific reaction that reduces O₂⁻ to H₂O₂ (see schema in Figure 1.4). In principle, any SOD-induced increase in I_p could be due to the elimination of Na⁺-K⁺ pump inhibition induced by O₂⁻ or it could be due to pump stimulation induced by H₂O₂. Since paraquat induces pump inhibition in the absence of exogenous SOD, the SOD-induced increase in I_p is more likely due to the elimination of O₂⁻ than due to the synthesis of H₂O₂. In

Chapter 3 it was concluded that endogenous synthesis of NO can mediate Na⁺-K⁺ pump stimulation. The results presented in this Chapter indicate that endogenous synthesis of O₂⁻ can mediate pump inhibition. The abolition of inhibition induced by paraquat by ROS/RNS scavengers, ebselen and SOD indicate strongly that ROS/RNS have a significant effect on cellular physiology. While a specific compound is not identified, it is likely that a RNS/ROS derivative of O₂ mediates the effect of paraquat on I_p by inducing an oxidative change on target molecules, perhaps even the Na⁺-K⁺ pump molecule itself. Thiol groups on cysteine residues on the Na⁺-K⁺ pump are particularly susceptible to oxidation. Reactions undergone include irreversible sulphinic or sulphonic oxidation, or, in the presence of glutathione, reversible S-glutathionylation, the most frequent form of oxidative thiol modification (Turko and Murad, 2002)(See discussion later in Chapter 7). The Na⁺-K⁺ pump's α and β subunits and the FXYD proteins all contain cysteine residues, and inhibition of isolated Na⁺-K⁺ ATPase exposed to oxidant stress *in vitro* has been attributed to oxidation of thiol groups (Sato et al., 1997). While the present work has not demonstrated oxidation of Na⁺-K⁺ pump subunits, it has shown that activity of the *in situ* Na⁺-K⁺ pump can be altered by oxidant stress sourced in the myocyte. Such an endogenous source of oxidant stress is required if ROS are to be considered as key mediators in physiological regulation of Na⁺-K⁺ pump function. This hypothesis will be examined further in Chapter 5.

Chapter 5

Ang II inhibits the cardiac Na-K pump via PKC and NADPH oxidase

5.1. NADPH oxidase as an endogenous source of $O_2^{\cdot-}$ in Angiotensin II induced Na^+-K^+ pump inhibition

The Na^+-K^+ pump is regulated by a variety of hormones, but the mechanisms that link their membrane surface receptors to pump function are poorly understood. Many receptors are coupled to activation of protein kinases, hence direct phosphorylation of the pump molecule has been proposed as a mechanism of pump regulation. However, phosphorylation sites are poorly accessible (Sweadner and Feschenko, 2001) and kinase activation is not necessarily associated with phosphorylation of the pump molecule (Cornelius et al., 2001). Phosphorylation that does occur may be to a very low stoichiometry (Cornelius and Mahmmoud, 2003b). The physiological role of phosphorylation of the Na^+-K^+ pump molecule in its regulation is therefore uncertain.

A group of small, single transmembrane proteins referred to as FXYD proteins have also recently been implicated in pump regulation (Garty and Karlish, 2006). However, with the exception of phospholemman (PLM or FXYD1), the FXYD proteins expressed in most mammalian tissues do not have functional phosphorylation sites. While the presence or absence of associated FXYD proteins modulate Na^+-K^+ pump function, they cannot account for kinase-

dependent pump regulation in most tissues and alternative mechanisms should be considered.

Oxidative modification is a potential alternative mechanism of protein regulation (Adachi et al., 2004, Cohen and Adachi, 2006, Ghezzi, 2005). The $\text{Na}^+\text{-K}^+$ pump may be susceptible to such regulation because its subunits and the lipid membrane in which they are embedded contain residues that can be oxidized. In agreement with this, exposure of $\text{Na}^+\text{-K}^+$ ATPase-enriched membrane fragments to chemical oxidants reduces the ATPase activity (Ellis et al., 2003). .Also, oxidants produced by intrinsic cellular sources inhibit the $\text{Na}^+\text{-K}^+$ pump as indicated by a reduction in I_p when isolated cardiac myocytes are exposed to paraquat as discussed in Chapter 4. Paraquat “uncouples” NOS, compelling it to preferentially produce O_2^- . This finding suggested that a ROS/RNS-derived, receptor-coupled, kinase-activated cellular source may mediate $\text{Na}^+\text{-K}^+$ pump regulation.

Ang II-induced pump inhibition (Hool et al., 1996, Buhagiar et al., 2001b) may be mediated by ROS/RNS since Ang II activates NADPH oxidase (Toda et al., 2007), the main source of O_2^- in cardiac myocytes (Hingtgen et al., 2006). NADPH oxidase is of particular interest as a cellular source of O_2^- because it is membrane-associated and its activation is coupled to Ang II type 1 receptors (AT1) (Toda et al., 2007) (Section 1.4.3). It has previously been shown that Ang II inhibits the $\text{Na}^+\text{-K}^+$ pump (Buhagiar et al., 1999, Buhagiar et al., 2001a), therefore we can speculate that the inhibition may be mediated by Ang II-induced activation of NADPH oxidase. Since both Ang II-induced NADPH

oxidase activation (Lambeth et al., 2007) and sarcolemmal Na⁺-K⁺ pump inhibition (Buhagiar et al., 2001a) are PKC-dependent, the established role of PKC in pump regulation is readily accommodated by such a scheme. The role of PKC ϵ is further examined in this Chapter.

5.2. Methods and experimental solutions.

Patch pipette solutions contained 10 mM Na⁺. The superfusate contained Na⁺ or it was Na⁺-free (see section 2.3.3 for details of composition). The pipette solutions contained 10 μ M L-arg in all experiments (see section 2.3.4 for composition), as our earlier work suggested that this NOS substrate may be limiting in patch-clamped myocytes. Angiotensin II (Sigma, St Louis, Missouri, USA) in a concentration of 100 nM was dissolved in the superfusate solution prepared as described in section 2.3.3. Superoxide dismutase (SOD, bovine erythrocyte, (Sigma, St Louis, Missouri, USA) in a concentration of 200 IU/mL was dissolved directly in the pipette solution.

A selective NADPH oxidase inhibitor, apocynin (acetovanillone) (Sigma, St Louis, Missouri, USA), was used to examine the role of NADPH oxidase. Apocynin inhibits the assembly of the p47^{phox} subunit of NADPH oxidase with the membrane complex thereby rendering NADPH oxidase inactive and inhibiting the formation of O₂⁻ (Stolk et al., 1994). Apocynin was first dissolved in DMSO and then diluted in patch pipette solution to a final concentration of 10 μ M. The final concentration of DMSO in the solution was 0.03%. DMSO in this concentration has no effect on I_p (Gadsby and Nakao, 1989). Another selective

inhibitor of NADPH oxidase, gp91ds (gp91 docking sequence) peptide (Sigma-Genosys pharmaceuticals, NSW, Australia) was used as it is a competitive antagonist that prevents the binding of p47^{phox} and gp 91^{phox} subunits of NADPH oxidase, thus inhibiting its activation. Gp91ds was dissolved in water and included in pipette solution to a final concentration of 10 μ M.

A specific PKC inhibitor, ϵ PKC inhibitory peptide (Calbiochem, Darmstadt, Germany), was used to examine the interaction of kinases and Ang II. The ϵ PKC inhibitor peptide was dissolved in water and added to the pipette solution to a final concentration of 100 nM.

Paraquat (Sigma, St Louis, Missouri, USA) was dissolved in the pipette solution to a final concentration of 100 nM as described in section 4.1

5.3. Results.

To examine the effect of Ang II on Na⁺-K⁺ pump activity, patch-clamped myocytes were exposed to a control superfusate or a superfusate containing 100 nM Ang II. Myocytes were exposed to the Ang II containing superfusate after the whole-cell configuration had been established. Panel A in Figure 5.1 shows the timing of changes in the composition of superfusates. Panel B shows holding currents of a control myocyte and a myocyte exposed to Ang II before and after exposure to ouabain. The ouabain-induced shift in holding current, defining I_p , was smaller for the myocyte exposed to Ang II than for the control myocyte. The mean I_p for control myocytes and myocytes exposed to

Ang II are shown in panel C of Figure 1. Ang II induced a significant decrease in I_p .

While wide-tipped patch pipettes provide good control of intracellular Na^+ (William et al., 2005b), the electrochemical driving gradient for Na^+ is inward and Ang II might, in principle, decrease Na^+ influx and hence the intracellular Na^+ concentration, resulting in a secondary reduction in I_p . To examine if an Ang II-induced decrease in Na^+ influx can account for the decrease in I_p , and to support the conclusion that Ang II induces a decrease in intrinsic $\text{Na}^+\text{-K}^+$ pump current with an independent set of data, I_p was measured in Na^+ -free superfusates, again using the protocol shown in Panel A of Figure 5.1. Results are included in Panel C. Ang II induced a decrease in mean I_p similar to the decrease measured in the Na^+ -containing superfusate. The normalized holding currents recorded after exposure of myocytes to ouabain using Na^+ -free or Na^+ -containing superfusates in all experiments summarized in Figure 5.1 were similar, indicating that, under the conditions used in these experiments, Ang II did not induce a detectable change in non-pump membrane current.

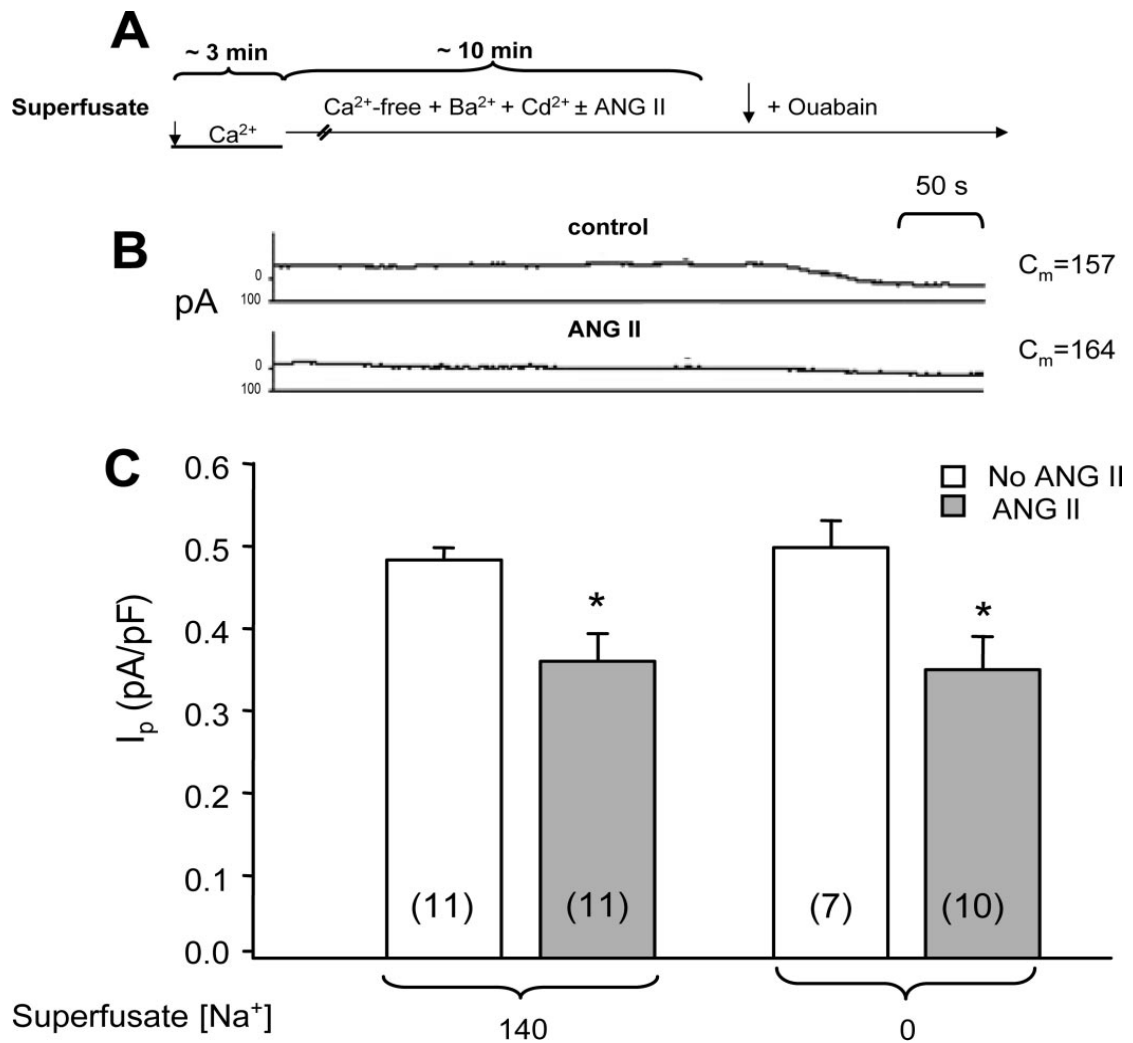


Figure 5.1 Effect of Ang II on Na⁺-K⁺ pump current I_p

Panel A shows the timing of changes in the composition of superfusates. The arrow on the left side of the panel indicates establishment of the whole-cell configuration and perfusion of the intracellular compartment with pipette solution. The switch from a Ca²⁺-containing, Ang II-free solution in the tissue bath to a nominally Ca²⁺-free solution containing Ba²⁺, Cd²⁺ and Ang II and the switch to a solution also containing ouabain (arrow on the right side of the panel) are shown. **Panel B** shows examples of holding current before and after exposure to ouabain. C_m indicates membrane capacitance in pF. **Panel C** shows mean I_p normalized for membrane capacitance measured in Na⁺-containing or Na⁺-free superfusates. Numbers of myocytes in each group are shown in parentheses. * indicates a significant difference between means of I_p.

Since Ang II activates NADPH oxidase (Griendling et al., 1994, Williams and Griendling, 2007), we examined the potential role of ROS in Ang II-induced pump inhibition by including 200 IU/ml SOD in patch pipette solutions. Superfusates contained Na^+ . Mean I_p for control myocytes and myocytes exposed to Ang II are summarized in Figure 5.2. Perfusion of the intracellular compartment with pipette solution containing SOD abolished the Ang II induced decrease in I_p .

The dependence of Ang II-induced pump inhibition on NADPH oxidase was examined next. In one series of experiments, 10 μM of the pharmacological inhibitor of NADPH oxidase, apocynin, was included in patch pipette solutions. Mean I_p s are included in Figure 5.2. Apocynin abolished the Ang II-induced decrease in I_p . In a second series of experiments 10 μM of the gp91ds peptide was included in patch pipette solutions to inhibit docking of p47^{phox} and hence activation of NADPPH oxidase. The gp91ds peptide is usually considered a low-efficacy inhibitor when combined with the *tat* peptide to facilitate transmembrane entry into intact cells (Bedard and Krause, 2007). However, the whole cell patch clamp technique allowed direct perfusion of the intracellular compartment with a *tat*-free gp91ds peptide. Mean I_p for control myocytes and myocytes exposed to Ang II are summarised in Figure 5.2. The gp91ds peptide abolished the Ang II induced decrease in I_p .

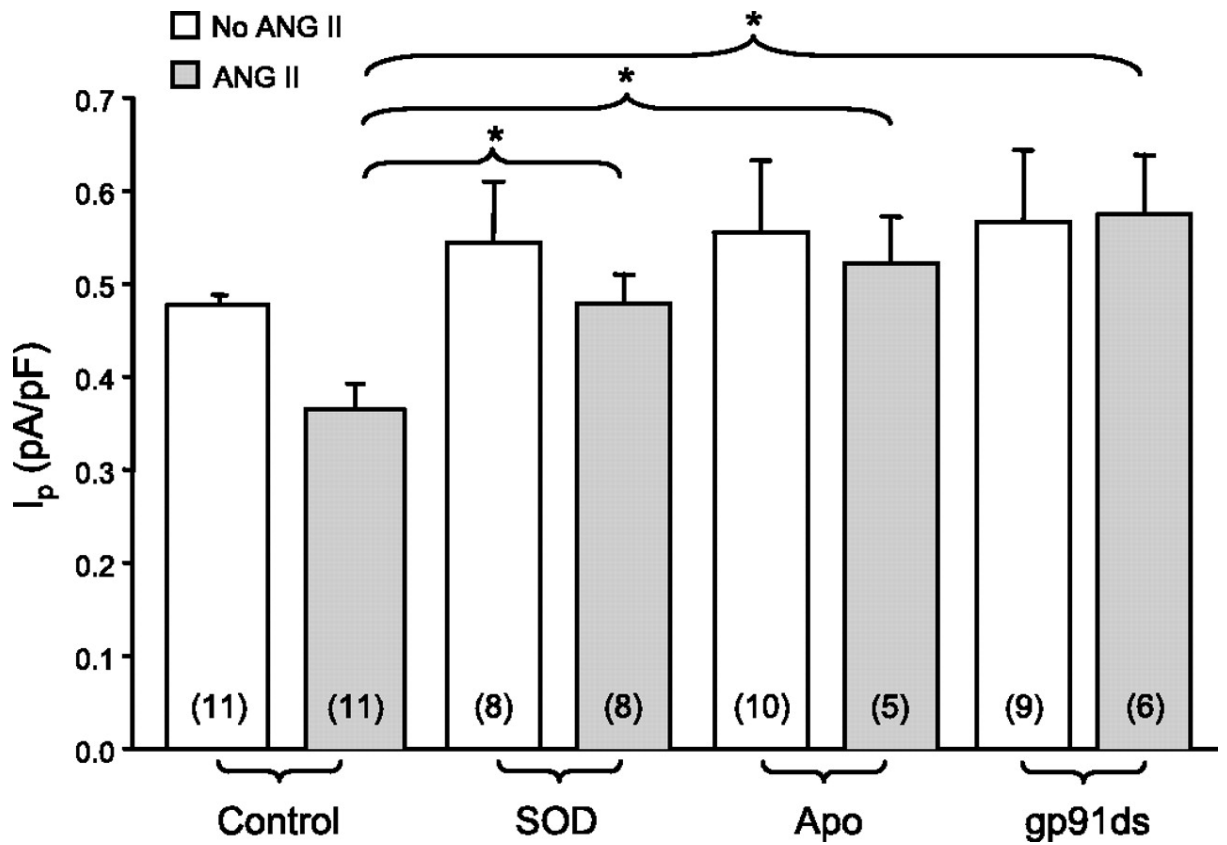


Figure 5.2 Role of superoxide and NADPH oxidase in Ang II-induced $\text{Na}^+\text{-K}^+$ pump inhibition

Myocytes were exposed to superfusates as shown in Panel A of Figure 5.1 and they were perfused with pipette solutions containing SOD, Apocynin (Apo) or the gp91ds peptide as indicated. Numbers of myocytes in each group are indicated in parentheses. * indicates a significant difference between means of I_p .

To examine the role of ϵ PKC in the Ang II-induced decrease in I_p , 100 nM of ϵ PKC inhibitory peptide was included in patch pipette solutions. Mean I_p for control myocytes and myocytes exposed to Ang II are presented in Figure 5.3. The ϵ PKC inhibitory peptide abolished the Ang II induced decrease in I_p .

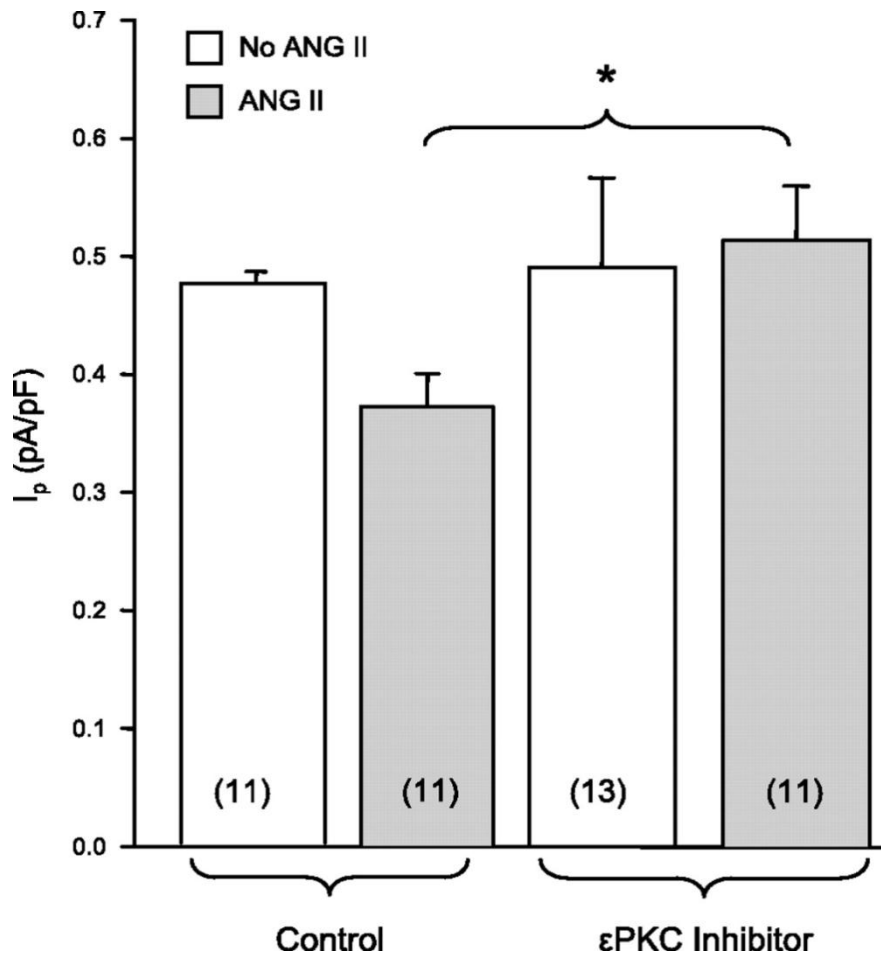


Figure 5.3 Role of PKC in Ang II-induced $\text{Na}^+\text{-K}^+$ pump inhibition

Myocytes were exposed to superfusates as shown in Panel A of Figure 5.1 and were perfused with pipette solutions containing ϵ PKC inhibitory peptide as indicated. Numbers of myocytes in each group are indicated in parentheses. * indicates a significant difference between means of I_p . Inhibition of ϵ PKC abolished Ang II-induced $\text{Na}^+\text{-K}^+$ pump inhibition.

Since oxidative signalling might, in principle, activate PKC, the Ang II- induced decrease in I_p might be due to $O_2^{\cdot-}$ -dependent activation of PKC and downstream phosphorylation of the Na^+K^+ pump itself or associated molecules. To examine this, oxidative stress was induced by including 100 μ M paraquat in pipette solutions. Myocytes were not exposed to Ang II. Mean I_p was significantly lower than mean I_p recorded in control experiments as shown in Figure 5.4. Inclusion of ϵ PKC inhibitory peptide in the patch pipette solution had no effect on the paraquat-induced decrease in I_p . These findings suggest that Ang II-induced pump inhibition is not due to direct phosphorylation of the pump molecule by $O_2^{\cdot-}$ -activated ϵ PKC and imply that ϵ PKC's position in the signalling cascade is proximal to the oxidant signal.

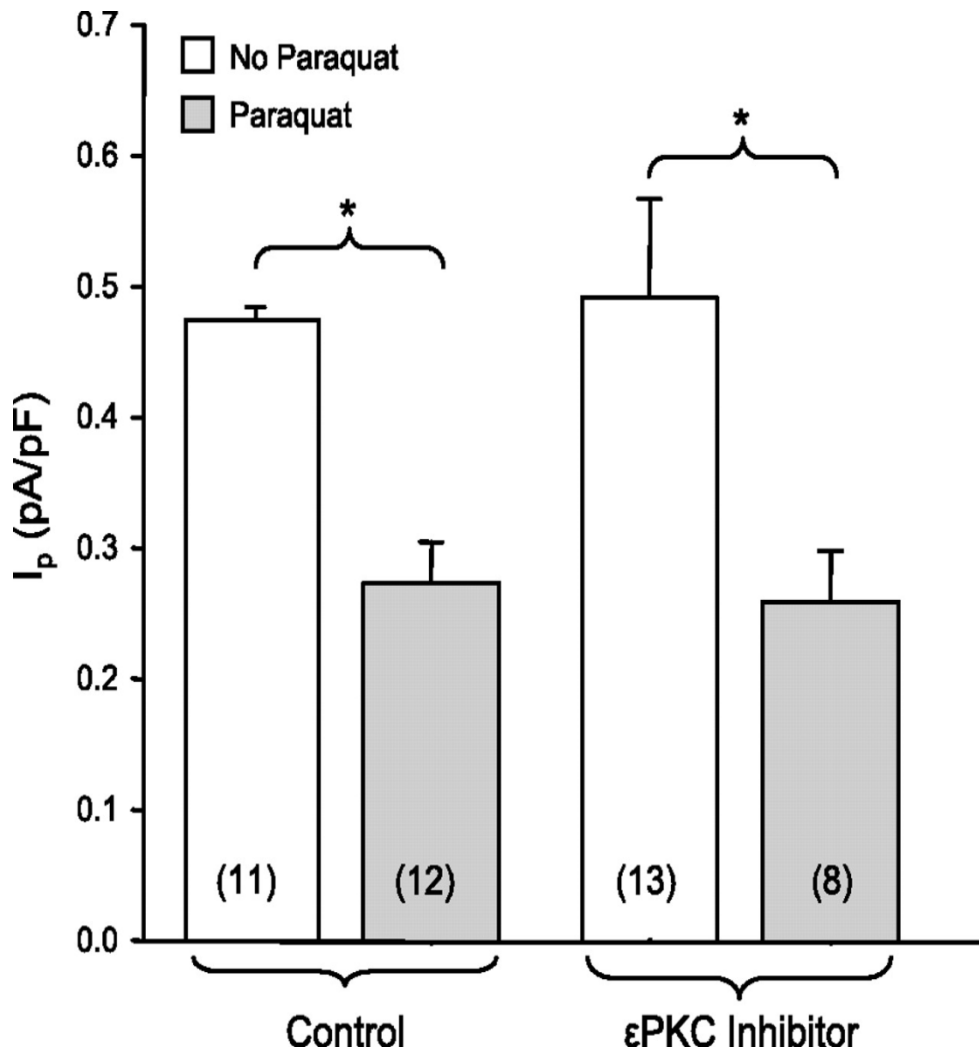


Figure 5.4 Oxidant stress and PKC-dependent $\text{Na}^+\text{-K}^+$ pump inhibition

Myocytes were perfused with pipette solutions that included paraquat and/or ϵ PKC inhibitory peptide as indicated. The ϵ PKC inhibitory peptide had no effect on the paraquat induced $\text{Na}^+\text{-K}^+$ pump inhibition. * indicates a significant difference compared to control. Numbers of myocytes in each group are indicated in parentheses.

5.4. Discussion.

The data presented in this Chapter indicates that ROS mediates Ang II-induced Na⁺-K⁺ pump inhibition. The abolition of the Ang II-induced pump inhibition by SOD implicates O₂⁻ in the pathway, thus linking Ang II receptors to the Na⁺-K⁺ pump. Similarly, the abolition of the Ang II-induced pump inhibition by apocynin suggests that NADPH oxidase is the source of O₂⁻. However, apocynin and all other pharmacological blockers have limited specificity for NADPH oxidase (Bedard and Krause, 2007). The gp91ds peptide, designed to specifically inhibit NADPH oxidase by mimicking its docking sequence with the p47^{phox} subunit was used. Perfusion of the intracellular compartment with pipette solution containing 10 μM of gp91ds peptide abolished the Ang II-induced decrease in I_p in good agreement with an IC₅₀ in the low micromolar range for NADPH oxidase inhibition in cell-free systems (Rey et al., 2001). This implicates the NOX family of oxidases in the pump inhibition.

5.5.1. Functional studies.

The functional studies using specific elimination of O₂⁻ with SOD, or inhibition of NADPH oxidase with the gp91ds peptide strongly implicate NADPH oxidase in Na⁺-K⁺ pump inhibition. Subsequent studies in our laboratory have been performed to facilitate the acceptance of the major paradigm shift . These studies aimed to examine if a physical association between NADPH oxidase and the Na-K⁺ pump exists. Such proximity between sources and targets of ROS is expected to facilitate ROS-dependent protein modification. Co-

immunoprecipitation experiments of the pump's α_1 subunits with NADPH oxidase subunits were performed. Results are shown in Figure 5.5. Experiments were performed by Dr C-C Liu and Dr G Figtree (White et al., 2009c)

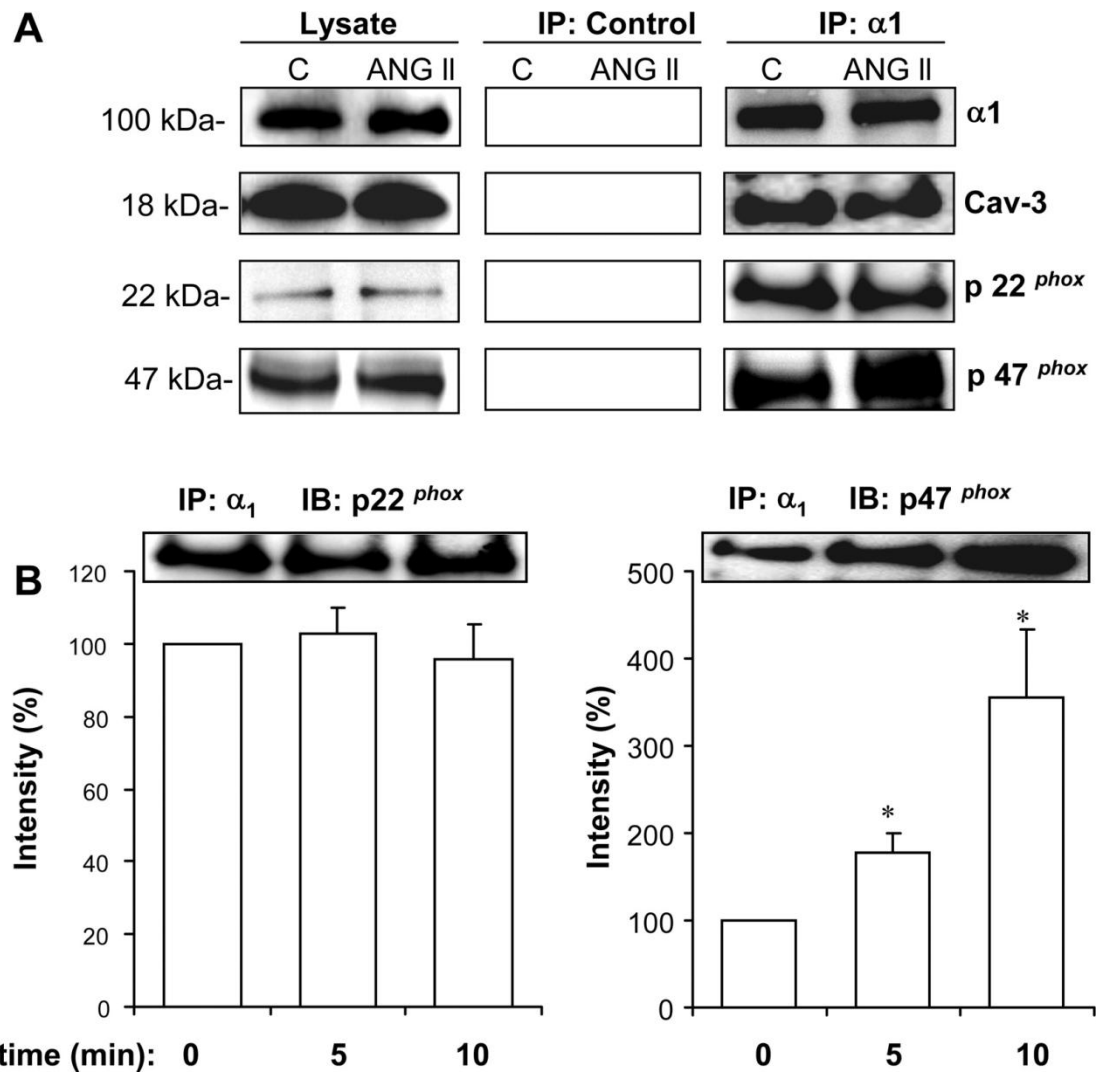


Figure 5.5 Co-immunoprecipitation of the Na⁺-K⁺ pump with caveolin and NADPH oxidase subunits

Co-immunoprecipitation of the Na⁺-K⁺ pump with caveolin and NADPH oxidase subunits (reproduced from White et al.). A. Cardiac myocytes were exposed to control solutions (C) or solutions containing Ang II. Cell lysates were immunoprecipitated with either anti- α_1 Na⁺-K⁺ pump subunit antibody (IP: α_1) or with nonimmune IgG₁ control antibody (IP: Control). Immunoprecipitates were resolved by denaturing SDS-PAGE, blotted, and then probed with monoclonal antibodies to caveolin 3 (Cav 3); p22^{phox} subunit, or p47^{phox} subunit. The immunoblots shown are representative of 3 independent experiments. B. Effect of duration of Ang II exposure on co-immunoprecipitation of α_1 subunit of Na⁺-K⁺ pump and NADPH oxidase subunits.

Representative immunoblots of p22^{phox} and p47^{phox} immunoprecipitated with antibody to α_1 subunit after either 0, 5, or 10 min exposure to Ang II. The mean intensity (mean gray level \pm SEM) of immuno-labelling is shown: for p22^{phox} on the left and p47^{phox} on the right. The association of α_1 subunit and the membrane subunit p22^{phox} is unaffected by exposure to Ang II. The co-immunoprecipitation of p47^{phox} and α_1 subunit of the pump is increased in a time-dependent manner. * represents significant difference compared with unexposed myocytes; n=3.

The α_1 subunits co-immunoprecipitated with the membrane-associated p22^{phox} and with the “cytosolic” p47^{phox} subunit that translocates to the membrane with NADPH oxidase activation. There was no effect of Ang II on α_1 /p22^{phox} co-immunoprecipitation but Ang II induced an increase in the α_1 /p47^{phox} co-immunoprecipitation. The α_1 subunits also co-immunoprecipitated with caveolin 3 (Yang and Rizzo, 2007) suggesting a caveolar microdomain for the interaction between the pump and NADPH oxidase. This is consistent with ϵ PKC inhibitory peptide abolishing the Ang II-induced changes in I_p since activated ϵ PKC localizes in cardiac myocyte caveolae (Rybin et al., 1999). Caveolar “signalosomes” that include membrane receptors, protein kinases and NADPH oxidase may mediate Na⁺-K⁺ pump inhibition. Since NOS also localizes in cardiac myocyte caveolae (Barouch et al., 2002, Liu et al., 2008) and mediates receptor-activated Na⁺-K⁺ pump stimulation (William et al., 2008b), the concept of such “signalosomes” can perhaps be expanded to include opposing receptor-coupled effects of NOS and NADPH oxidase to fine-tune Na⁺-K⁺ pump activity. However, it should be recognized that this simple scheme may be complicated

by an interaction with other sources of ROS because $O_2^{\cdot -}$ generated by NADPH oxidase can act as “kindling” for further $O_2^{\cdot -}$ synthesis by the uncoupling of nitric oxide synthase (Bedard and Krause, 2007) and by the activation of xanthine oxidase (Lerman and Lerman, 2007). The practical significance for signalling of such effects may ultimately depend on the physical proximity of sources and scavengers of ROS in relevant microdomains.

5.5.2. Ang II induces a decrease in pump affinity for Na^+ .

Although this study was performed on patch-clamped, internally perfused myocytes, previous studies indicate that Ang II also inhibits the pump in isolated intact myocytes and *in vivo* (Hool et al., 1995b, Hool et al., 1996, Hansen et al., 2007). In these studies, rabbits were treated with an angiotensin converting enzyme (ACE) inhibitor or an Ang II type I receptor (AT_1) antagonist for 1 week, myocytes were isolated and I_p was measured. Inhibition of Ang II signalling *in vivo* was associated with an increase in pump activity in isolated patch clamped myocytes and a decrease in the intracellular Na^+ concentration measured in isolated papillary muscles (Hool et al., 1995b). The increase in I_p was only detectable when patch pipette solutions contained Na^+ in a rate-limiting intracellular concentration (Buhagiar et al., 1999, Hool et al., 1996) and it was dependent on the inclusion of K^+ in pipette solutions. Exposure of myocytes to Ang II *in vitro* before (Hool et al., 1996, Buhagiar et al., 1999) or after (Buhagiar et al., 2001b, Buhagiar et al., 1999) the whole-cell configuration was established induced a K^+ -dependent decrease in I_p . Such K^+ -dependence is consistent with an increase in the rate constant for the Na^+ - K^+ pump's backward $E_1 + 2K^+ \rightarrow$

$E_2(2K^+)$ reaction, effectively reducing its selectivity for Na^+ (Buhagiar et al., 2004). This effect is a good candidate for regulation since the $E_2 \rightarrow E_1$ conversion is a major rate-limiting step in the pump's reaction cycle (Lupfert et al., 2001). When taken together, the studies indicate that Ang II induces a physiologically relevant decrease in the pump's selectivity for intracellular Na^+ . The role of PKC in Ang II induced Na^+-K^+ pump inhibition described in this Chapter is at odds with findings in mouse ventricular myocytes. Han et al., (Han et al., 2006) reported that PKC increases the Na^+-K^+ pump's maximal activity, but has no effect on its Na^+ affinity. In the studies in this Chapter, PKC was implicated by the use of an isoform-specific inhibitory peptide, while in the mouse studies PKC was implicated by the non-specific activation of PKC with the phorbol ester PDBu (Han et al., 2006). However, this is unlikely to account for the discrepancy because the effects of PKC activation with a phorbol ester or the ϵ PKC activating peptide, $\psi\epsilon$ RACK are similar. Both induced Na^+-K^+ pump inhibition (Hool et al., 1996, Buhagiar et al., 1999). There are other variations between the studies by Han et al and those detailed in this chapter that may account for the differences in findings and these are reviewed in detail below.

The study by Han et al (2006) reported that activation of PKC with the phorbol ester PDBu stimulates the Na^+-K^+ pump in cardiac myocytes. Stimulation was attributed to phosphorylation of PLM since PDBu did not stimulate the pump in myocytes from PLM knock-out mice. The Na^+ -sensitive fluorescent dye SBFI was used to measure the rate of decline of intracellular Na^+ after a period of Na^+ -loading in K^+ -free solutions. SBFI has a long track record of technical

problems, including difficulties with calibration of the signal. Han et al calibrated their signal up to 20 mM intracellular Na^+ (Figure 1A in their paper) but extrapolated measurements up to 50 mM (Figure 1B). The conclusion that PDBu increases V_{max} by ~60% is critically dependent on extrapolation to the high end of the concentration range. Indeed, one could argue that an even higher concentration than 50 mM would have been needed to allow the fit of the Hill equation to the data in Figure 1B (in their paper) with appropriate constraints.

Han et al (2006) used simultaneous measurements of pump current and intracellular Na^+ (SBFI) of patch clamped myocytes to independently support the data in their Figure 1. Results seem strange. Small patch pipettes were used and myocytes were Na^+ -loaded by exposing them to 0 mM K^+ for a period. The pump was then re-activated by exposure to 4 mM K^+ . This is a modification of protocols that have been used by many other groups in the past. The well-justified tradition has been to determine the rate constant for the decline of the outward pump current after K^+ activation. A logarithmic transformation of K^+ -activated I_{pump} for WT before and after exposure to PDBu in Figure 3A suggests that there is minimal effect of PDBu on the rate constant for current decline in the particular example shown. In contrast, PDBu induces a substantial *increase* in the same rate constant for the PLM-KO myocyte shown in Figure 3D. This is difficult to understand since the paper concludes that PLM mediates the effect of PKC activation.

The SBFI measurements in Fig 3 differs from the one shown in Figure 1A, (PLM-KO) by the way SBFI was introduced into the myocytes (pre-loading in Figure 1 vs. inclusion in pipette solution in Figure 3). There was no effect of PDBu on the rate of decrease of the SBFI signal in Figure 1A while PDBu induced a marked increase in this rate for the PLM-KO myocyte shown in Figure 3E. Regardless of the interpretation of the derived data in Figure 3C/F, Figure 3E makes it difficult to escape the conclusion that PDBu increases pump activity in PLM-KO myocytes if one accepts that the SBFI measurements accurately reflect pump activity. This is the exact opposite of the overall conclusion of the paper.

Han et al used “low-resistance” pipettes containing 100 mM Na⁺ to saturate intracellular pump sites and independently verify that PDBu increased V_{max} in WT myocytes (page 1379). “Low resistance” was defined by an initial resistance ≤ 2 M Ω , and a series resistance < 5 M Ω . PDBu induced an increase in I_{pump} but only by 10% (see page 1381), in stark contrast to the 60% determined using the SBFI data shown in Figures 1 and 2. The authors appreciated that this was a serious problem, and they offered the explanation that PKC was dialysed out of the cell when large pipettes were used. *A priori*, this seems an unlikely scenario since signalling molecules do not float around freely in the cytosol; they are anchored.

Additional experiments were performed to support the “dialysis hypothesis”. PLM phosphorylation was measured when cells were permeabilized to allow PKC to escape *via* a route other than patch pipettes. A reduction in the level of

PDBU-induced phosphorylation in the permeabilized myocytes was taken as support for the idea that PKC had been dialyzed out of cells in the patch clamp experiments (page 1379). However, permeabilization was achieved by exposing myocytes to saponin. This is likely to affect lipophilic enzymes, including PKC, and also skin the sarcolemmal membrane in which PLM is embedded. It is therefore not surprising that the amount of phosphorylated PLM is reduced in saponin-permeabilized cells. It is difficult to imagine a more inappropriate experiment to support the PKC dialysis hypothesis the authors propose.

Han et al. claim a consistency in their data by the finding that an ~50% higher V_{max} in PLM-KO myocytes (when expression level is taken into account) compares well to the PDBu-induced rise in V_{max} in WT myocytes (page 1381). A simple scheme of phosphorylation-induced relief from PLM-inhibition of the pump in WT is taken to account for the match. However, as pointed out in an accompanying editorial (page 1290-1292), a study on PLM-KO mouse heart by another group Jia et al. (Jia et al., 2005) reported an ~50% *decrease* in V_{max} in PLM-KO compared with WT. It is very difficult to reconcile these findings with the study by Han et al. The consistency of data and the simple scheme Han et al. propose is not applicable if one chooses to accept the findings in the study by Jia et al. The editorial suggested the discrepancy reflected how scared mice were in the two different studies. That is not a rigorous interpretation.

In the Discussion, Han et al. claim there some consensus that PKC activation enhance the activity of the Na^+K^+ pump and refers to previous studies (Gao et

al., 1999, Wang et al., 1998, Erlenkamp et al., 2002). All the papers they refer to used patch pipettes that almost certainly were too small to control intracellular Na^+ and series resistances were not reported. None of them showed stimulation of I_{pump} in experiments where PKC-mediated Na^+ influx and secondary pump stimulation was ruled out by use of Na^+ -free extracellular solutions. Han et al. must have been aware of the need for such control experiments since they report that PKC activation induces a large increase in Na^+ influx (see Figure 6C, page 1382 in their paper). The consensus they claim may well just reflect a consensus on the fact that PKC activation induces Na^+ influx that is detected as a secondary increase in the " I_{pump} " that is reported. This possibility should have been ruled out.

Phosphorylation of the pump related FXYD protein PLM has been implicated as a mechanism for PKC dependent pump regulation (Cornelius et al., 2001, Garty and Karlish, 2006, Han et al., 2006, Bibert et al., 2008). However, it is unlikely that phosphorylation of PLM mediates the Ang II induced pump inhibition in the experiments reported in this Chapter because inhibition was abolished by SOD and the gp91ds peptide, compounds not expected to inhibit PKC. The finding that Ang II inhibits the pump via NADPH oxidase provides a novel explanation for PKC-dependent pump regulation, since PKC's role in Ang II induced phosphorylation of p47^{phox} and subsequent activation of NADPH oxidase is firmly established (Williams and Griendling, 2007). It is a strength of this scheme that it accounts for pump regulation in tissues not expressing FXYD proteins with phosphorylation sites. However, it is important for its credibility that there is a downstream molecular effect on the $\text{Na}^+\text{-K}^+$ pump that can alter

function and is stable, yet reversible. Of the known oxidative modifications of proteins, glutathionylation fulfils these criteria. Glutathionylation is the formation of a mixed disulfide bond between the intracellular tripeptide glutathione, and a candidate reactive cysteine residue on the target protein (Ghezzi review; Adachi et al 2004). Oxidative stimuli induce glutathionylation, a reversible oxidative modification, of Cys46 of the β_1 subunit (Figtree et al., 2009a). Glutathionylation and an associated pump inhibition are abolished by mutation of Cys46 indicating a causal relationship between the oxidative modification and pump function. This is supported by the absence of an effect of oxidative stimuli when expressed pump heterodimers contain wild-type β_2 or β_3 subunits that do not have a Cys46 (Figtree et al., 2009a). The physiological relevance of glutathionylation is suggested by its coupling to hormone receptors and kinase-dependent signalling pathways. Exposure of cardiac myocytes to Ang II increases glutathionylation and inhibits pump activity via protein kinase C (PKC)-dependent activation of NADPH oxidase (White et al., 2009b, Figtree et al., 2009b). Conversely, exposure of myocytes to β_3 adrenergic receptor agonists decreases glutathionylation from baseline and stimulates pump activity via activation of nitric oxide synthase (NOS) and nitric oxide-dependent downstream pathways (Garcia et al., 2008). Thus, pump stimulation and inhibition are mediated by decreasing or increasing the degree of Cys46 glutathionylation from baseline. Recent studies in our laboratory have shown that chemically induced oxidant stress or Ang II-induced activation of NADPH oxidase induces glutathionylation of the pump's β_1 subunit and alters its interaction with the catalytic α_1 subunit (Liu et al., 2008, Figtree et al., 2009a). These findings will be described in more detail in Chapters 6 and 7. It is

proposed that glutathionylation of the pump's β_1 subunit accounts for the Ang II induced pump inhibition described in this Chapter.

Ang II-induced $\text{Na}^+\text{-K}^+$ pump inhibition mediated by redox signalling may be important for our understanding of the role of NADPH oxidase-dependent redox signalling in the pathogenesis of cardiac hypertrophy and failure (Murdoch et al., 2006). Raised cardiac myocyte levels of Na^+ are believed to contribute to the electro-mechanical phenotype of contractile abnormalities and arrhythmias in these conditions (Pieske et al., 2002), and the beneficial effect of ACE inhibitors may be due to the specific targeting of detrimental redox signalling (Seddon et al., 2007). The studies presented here suggest that the effects of such targeting on the $\text{Na}^+\text{-K}^+$ pump, and hence intracellular Na^+ levels, plays a role in this scheme. The effect of treatment with ACE inhibitors on the $\text{Na}^+\text{-K}^+$ pump has not been studied in heart failure. However, the effect of AT_1 blockade has been examined in a model of diabetes, a condition predisposing to heart failure and often associated with Ang II-induced NADPH oxidase activation (Lastra and Manrique, 2007). Treatment with losartan abolished sarcolemmal $\text{Na}^+\text{-K}^+$ pump inhibition associated with diabetes (Hansen et al., 2007), a finding in good agreement with a role of the pump as an effector molecule in conditions with NADPH oxidase-dependent redox signalling. The role of Ang II in the process of ROS/RNS generation via NADPH oxidase and its effect via these mediators on the $\text{Na}^+\text{-K}^+$ pump has been demonstrated in this Chapter.

Chapter 6

NADPH oxidase and β -adrenergic regulation of the $\text{Na}^+\text{-K}^+$ pump.

6.1 Introduction

Data presented in Chapter 5 demonstrated that Ang II, a neurohormone dysregulated in heart failure and other forms of cardiovascular disease, inhibits the $\text{Na}^+\text{-K}^+$ pump via oxidative signalling and PKC. This Chapter examines the regulation of the $\text{Na}^+\text{-K}^+$ pump by cyclic AMP-dependent messenger pathways which activated by β -adrenergic receptor agonists, are also classically dysregulated in heart failure.

Cyclic AMP-dependent messenger pathways have been reported to regulate the $\text{Na}^+\text{-K}^+$ pump. Such regulation occurs in many different tissues (Therien and Blostein, 2000), including the heart (Kockskämper et al., 2001, Gao et al., 1992, Gao et al., 1998), and is reported to cause either pump stimulation or inhibition. Most previous studies have reported that activation of PKA-dependent pathways stimulates the $\text{Na}^+\text{-K}^+$ pump by increasing its maximal rate or its affinity for intracellular Na^+ (Despa et al., 2005, Bibert et al., 2008). Widely varying experimental conditions may have caused different steps in the complex reaction cycle to be rate-limiting. Of the many identified partial reactions, the $\text{E}_2 \rightarrow \text{E}_1$ conformational change and phosphorylation of the enzyme by ATP are important rate-determining steps under physiological conditions, whereby the rate of the phosphorylation reaction depends on the degree of saturation of the intracellular Na^+ binding sites. Both of these reactions are inhibited by

intracellular K^+ that competes with Na^+ for binding and accelerates the backward $E_1 \rightarrow E_2$ reaction and modest changes in the intracellular K^+ concentration can have large effects on forward pump rate. These and other complexities (Kong and Clarke, 2004) make it difficult to define and measure a “Na affinity” that has a meaningful mechanistic interpretation in intact cells and they have not been taken into account in studies on the effect of PKA-dependent pathways on the Na^+ - K^+ pump.

Glutathionylation of the β_1 subunit decreases the rate of the $E_2 \rightarrow E_1$ conformational change as indicated by studies on isolated Na^+ - K^+ ATPase (Figtree et al., 2009a). In principle, this can decrease maximal pump rate, or, because Na^+ binds to the E_1 configuration, it can appear to decrease the sensitivity of the pump to intracellular Na^+ in intact cells. However, regardless of experimental conditions, it cannot account for PKA-dependent Na^+ - K^+ pump stimulation reported by most previous studies. Experimental activation of cAMP-dependent pathways in previous studies on the cardiac myocyte Na^+ - K^+ pump has been achieved by exposing myocytes to a β adrenergic receptor agonist or by directly activating adenylyl cyclase with exposure to forskolin. Receptor activation is the most physiologically relevant of the two approaches. However, available agonists have poor selectivity against the 3 different β adrenergic receptor subtypes (Hoffmann et al., 2004, Mihailidou et al., 2000), and the compounds used in some previous studies may have activated the β_3 adrenergic receptor that is coupled to activation of NOS. We have found the β_3 adrenergic receptor mediates stimulation of the Na^+ - K^+ pump in cardiac myocytes (Garcia et al., 2008). We therefore use forskolin to activate adenylyl cyclase, as have most other recent investigations in this area. (Kockskamper et

al., 2000, Kockskamper et al., 2001, Erlenkamp et al., 2002, Silverman et al., 2005, Fuller et al., 2009).

As discussed in Chapter 5, exposure of cardiac myocytes to Ang II inhibits I_p via ϵ PKC dependant activation of NADPH oxidase. This signalling pathway is mediated by the glutathionylation of the β_1 subunit of the Na^+ - K^+ pump (White et al., 2009a, Figtree et al., 2009a). In this Chapter we show that the adenyl cyclase activator forskolin inhibits Na^+ - K^+ pump activity via a pathway that involves NADPH oxidase and superoxide. We show that crosstalk between PKA and ϵ PKC is critical to signalling.

6.2. Methods and experimental solutions.

Because activation of β_1/β_2 -adrenergic receptors is reported to induce an increase in I_p , most experiments were performed using Na^+ -free superfusates at the time the ouabain-induced shift in holding current was determined. This eliminated the possibility that alteration in I_p could be secondary to activation of transmembrane Na^+ -influx and an increase in the intracellular Na^+ concentration that in turn would stimulate the pump. Patch pipette solutions contained 10 mM Na^+ in most experiments. When indicated they contained 80 mM Na^+ . All superfusates used were Na^+ -free except when pipette solutions contained 80 mM Na^+ (see sections 2.3.3 and 2.3.4 for details of the composition of solutions).

Since it could not be ascertained that any agonist would be perfectly specific for β_1/β_2 -adrenergic receptors without any effect on the β_3 -adrenergic receptor,

forskolin was used in all experiments to activate adenylyl cyclase (AC). AC is the enzyme immediately downstream from β_1/β_2 -adrenergic receptors and universally activated when agonists bind to the receptors. Forskolin is a diterpene which is a ubiquitous activator of eukaryotic AC. Activation of AC results in the formation of cAMP and the subsequent activation of PKA. Forskolin is highly specific for AC and is often used experimentally as a “surrogate” for receptor activation for *in vitro* studies of $\beta_{1/2}$ -adrenergic signalling. When indicated, 100 nM forskolin was included in the Ba^{2+} -containing, Ca^{2+} -free superfusate always used after the whole-cell configuration had been established.

Apocynin was included in the superfusate in a concentration of 10 μM in some experiments to inhibit NADPH oxidase. Apocynin was first dissolved in DMSO. The solution was then diluted in superfusate to achieve the final concentration. The final concentration of DMSO in the superfusate was 0.01%. The PKA inhibitor H-89, dissolved in DMSO, was included in pipette solutions in a concentration of 10 μM when indicated. The final concentration of DMSO in the pipette solution was 0.3%. When indicated, the ϵ PKC-inhibitory peptide was included in pipette solutions in a concentration of 100 nM as described in Chapter 5, and SOD was included in pipette solutions in a concentration of 200 IU/mL as described in Chapter 4. The gp91ds peptide was included in pipette solutions in a concentration of 10 μM to inhibit NADPH oxidase as described in Chapter 5. Most experiments were performed using L-arg free pipette solutions. However, in a subset of experiments, the solutions contained 10 μM L-arg, as indicated in text and Figures.

Exposure of myocytes for forskolin and hence activation of an adenylyl cyclase/cAMP/PKA pathway is expected to activate a large Cl^- current (Bahinski et al., 1989). Since this might interfere with measurements of I_p , the test potential was set at the equilibrium potential for Cl^- (E_{Cl}) calculated from the concentrations of Cl^- in the pipette solution and the superfusate according to the Nernst equation:

$$E_{\text{Cl}} = RT/nF \ln[\text{Cl}]_o/[\text{Cl}]_i$$

where R is the universal gas constant, T is the absolute temperature in °Kelvin, n is the valence of the ion and $[\text{Cl}]_o$ and $[\text{Cl}]_i$ are the extracellular and intracellular concentrations of Cl^- respectively. A calculated E_{Cl} of -14 mV for the conditions of the experiments was used as the holding potential in all experiments.

6.3. Results.

The effect of forskolin on pump current was first examined by patch clamping myocytes using pipette solutions that included 10 mM Na^+ . The superfusate was Na^+ -free and pump current was measured after a patch clamped myocytes had been exposed to 100 nM forskolin included in the superfusate for ~5 min. Since cAMP-activated Cl^- currents are large, any Cl^- current induced by forskolin should be easy to identify as a difference in net membrane current

between the control myocyte and the myocyte exposed to forskolin after exposure to ouabain in the superfusate. The currents were very small and their means were similar for myocytes exposed or not exposed to forskolin. Figure 6.1 shows mean I_p measured in myocytes exposed to a forskolin-free control superfusate and forskolin-containing superfusate. Forskolin induced a significant decrease in mean I_p . Since the forskolin-induced decrease in I_p is not compatible with previously publications which concluded that PKA stimulated the $\text{Na}^+\text{-K}^+$ pump (Silverman et al., 2005, Despa et al., 2005), an independent set of experiments were performed to confirm results. The compositions of pipette solutions and superfusates were identical to those used in the first set of experiments except that 10 μM L-arg was included in pipette solutions. This was to ensure that L-arg, the key substrate for NOS, was not limiting, as well as to re-examine the effect of forskolin on I_p under separate experimental conditions. L-arginine is of particular interest since either a decrease or an increase in its concentration may promote oxidation: a reduction in L-arginine levels may uncouple NOS to preferentially synthesize superoxide rather than NO as discussed in Chapter 3. Conversely, NO synthesized from supplemental L-arginine may combine with superoxide to form the highly oxidant species peroxynitrite. To minimize risk of experimental artefact resulting from alterations in intracellular L-arginine concentrations, initial experiments were performed with and without L-arginine included in patch pipette solutions. In a first series of experiments we included 10 $\mu\text{mol/L}$ L-arginine in pipette solutions, a concentration ~ 10 -fold above the K_D for relevant NOS isoforms (White et al., 2008). The myocytes were superfused with forskolin-free “standard” Na^+ - and Ca^{2+} -containing Tyrode’s solution while the whole-cell

configuration was established. We then switched to a nominally Na^+ -free superfusate to rule out influx of Na^+ that might cause secondary pump stimulation. This superfusate was also nominally Ca^{2+} -free. It contained forskolin or was forskolin-free in control experiments. The means of I_p measured in myocytes exposed to a forskolin-free control superfusate and forskolin-containing superfusate are included in Figure 6.1. Figure 6.1A shows the timing of changes in the composition of superfusates. Figure 6.1B shows currents of a control myocyte and a myocyte exposed to forskolin. I_p was smaller for the myocyte exposed to forskolin than for the control myocyte. Figure 6.1C shows that forskolin induced a statistically significant decrease in mean I_p . An independent set of experiments was performed using an identical protocol with the exception that pipette solutions did not contain L-arginine. Forskolin induced a significant decrease in mean I_p similar to that seen without supplemental L-arginine. Holding currents after Na^+ - K^+ pump blockade with ouabain in both sets of experiments were very small and similar for control myocytes and myocytes exposed to forskolin, consistent with use of the calculated E_{Cl} as the test potential. Although qualitatively similar, the forskolin-induced decrease in I_p appeared larger when L-arginine was included in pipette solutions. To avoid a bias in favour of Na^+ - K^+ pump inhibition that we expected from the β_1 subunit glutathionylation and to facilitate comparisons with previous studies, we used patch pipette solutions without added L-arginine in all subsequent experiments.

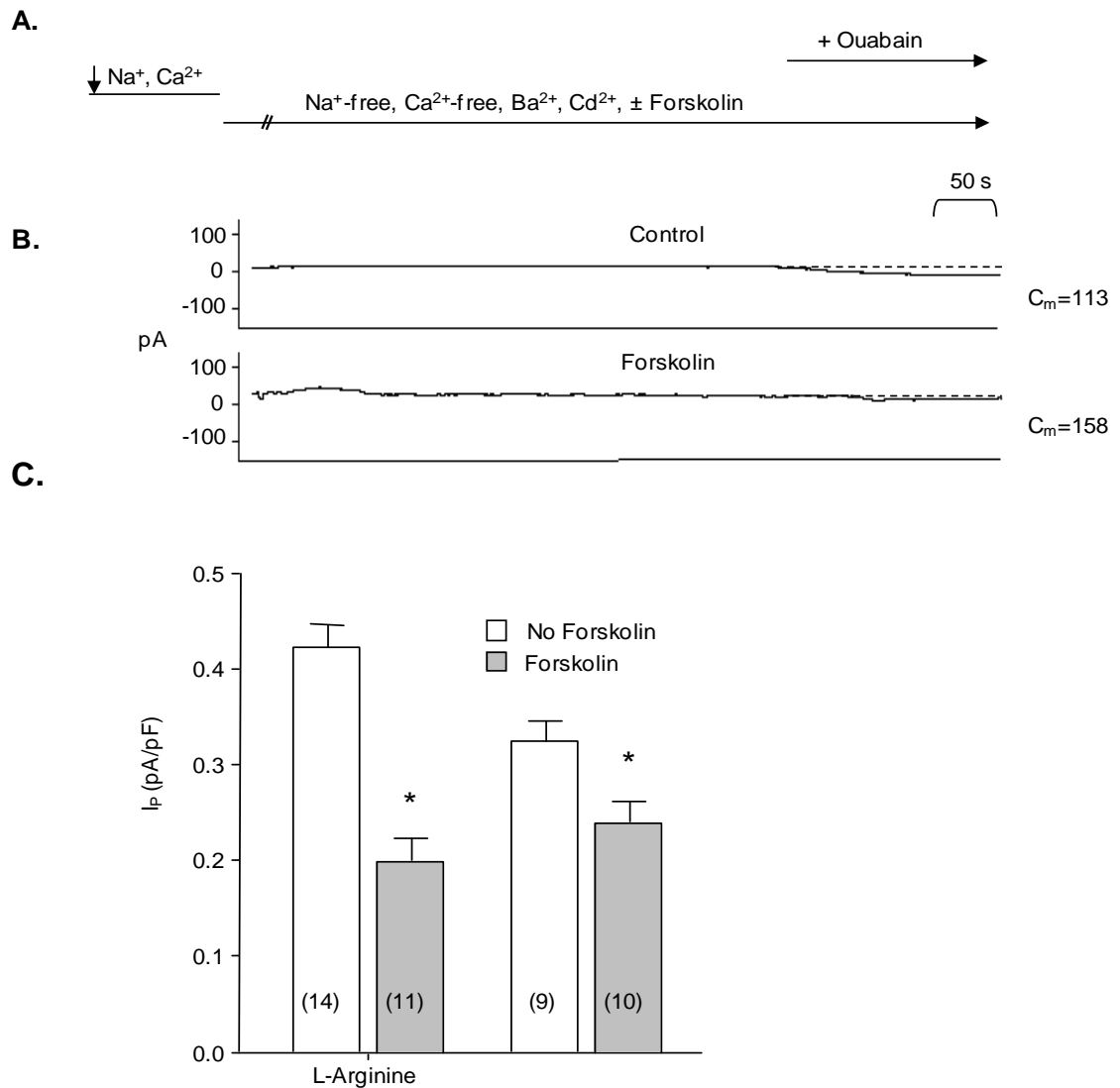


Figure 6.1 Effect of forskolin on $\text{Na}^+\text{-K}^+$ pump current in myocytes

Panel A shows the timing of changes in the composition of superfusates. The arrow on the left side of the panel indicates establishment of the whole-cell configuration and hence perfusion of the intracellular compartment with pipette solution. The switch from a Ca^{2+} -containing, forskolin-free solution in the tissue bath to a nominally Ca^{2+} -free solution containing Ba^{2+} , Cd^{2+} and forskolin, and the switch to a solution also containing ouabain (arrow on the right side of the panel) are shown. **Panel B** shows examples of holding currents. Large changes in the currents that occur the first 1-2 min after the switch to Na^+ - and Ca^{2+} -free, Ba^{2+} - and Cd^{2+} -containing superfusate, before currents stabilize, are not shown. Stable holding currents before exposure of myocytes to ouabain are important for the measurement of I_p . Stability of the currents as well as the

ouabain-induced shift in them was identified from the read-out of an electronic cursor. C_m indicates membrane capacitance in pF. **Panel C** shows mean I_p normalized for membrane capacitance. Pipette solution contained L-arginine where indicated. Numbers of myocytes in each group are indicated in parentheses. * indicates a significant difference between means of I_p .

6.3.1 Does Forskolin affect Na⁺-K⁺ pump turnover?

To examine if forskolin induces a decrease in maximal Na⁺-K⁺ pump turnover rate, myocytes were voltage clamped using pipette solutions that included 80 mM Na⁺, a concentration expected to nearly saturate intracellular pump binding sites. Since it is technically difficult to maintain the whole-cell configuration in a Na⁺-free superfusate when the intracellular Na⁺ concentration is high, Na⁺-containing (140 mM) superfusates were used. Because of the high Na⁺ concentration in pipette solutions there was no net inward electrochemical gradient for Na⁺ despite the presence of Na⁺ in the superfusate. Pipette solutions did not contain L-arg. I_p was measured with and without 100 nM forskolin included in the superfusate. Forskolin had no effect on the holding current recorded after myocytes had been exposed to ouabain indicating that forskolin did not induce a change in non-pump membrane currents under the conditions of the experiments. Mean I_p measured in myocytes exposed to a forskolin-free control superfusate and forskolin-containing superfusate is shown in Figure 6.2. Forskolin had no significant effect on mean I_p when pipette solutions included 80 mM Na⁺.

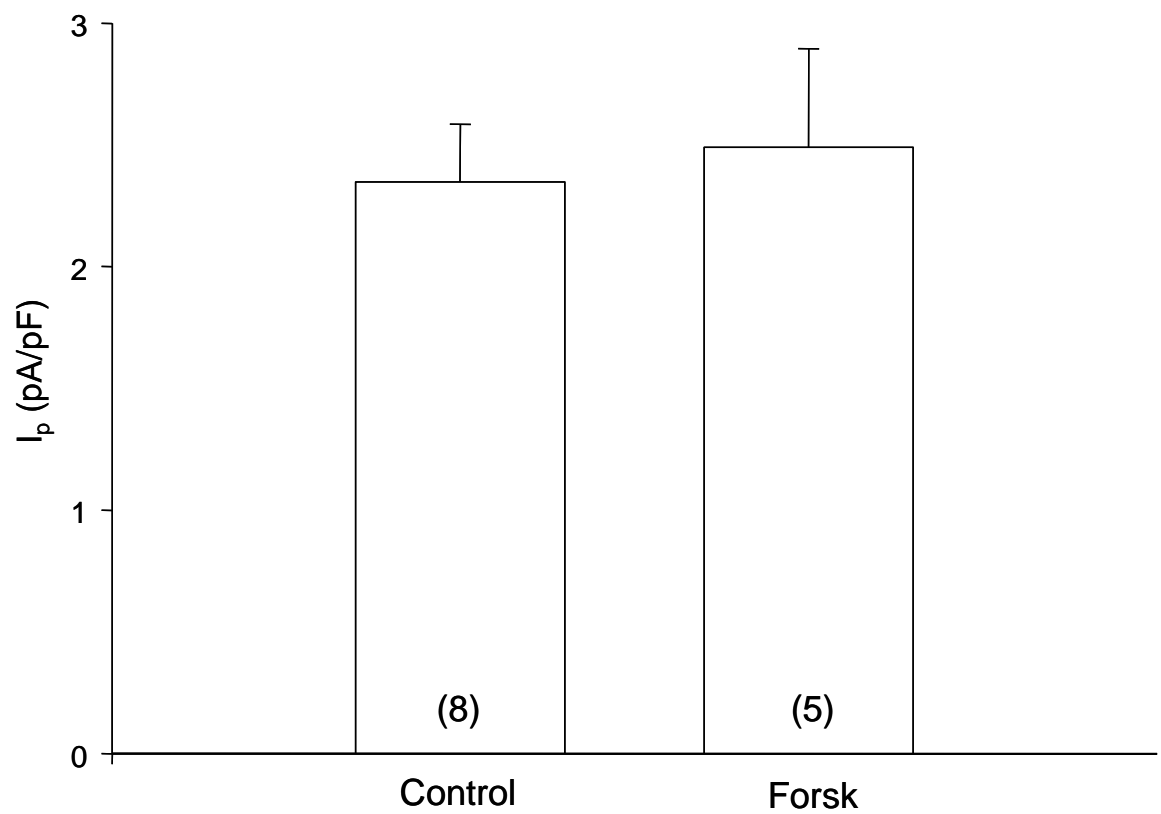


Figure 6.2. Effect of forskolin on near-maximal I_p

The pipette Na^+ concentration was near saturating for intracellular pump sites (80 mM). The superfusate contained Na^+ . The test potential was -14 mV. Numbers in parentheses indicate number of experiments.

6.3.2 Role of superoxide in forskolin-induced Na⁺-K⁺ pump inhibition

As described in Chapter 5, exposure of cardiac myocytes to Ang II-induced a decrease in I_p . This decrease was mediated by $O_2^{\cdot-}$ produced by NADPH oxidase (Figure 5.2). Experiments were performed to examine if $O_2^{\cdot-}$ mediates the forskolin-induced decrease in I_p . The effect of SOD on the forskolin induced decrease in I_p was examined in an initial series of experiments. Patch pipette solutions included 10 mM Na⁺ and 200 IU/mL of SOD to eliminate $O_2^{\cdot-}$. The superfusate was Na⁺-free and it contained 100 nM forskolin or it was forskolin-free. Results are shown in Figure 6.3. SOD had no effect on I_p recorded in the forskolin-free control superfusate. However, the addition of SOD to the pipette solution abolished the forskolin-induced decrease in mean I_p , that had been recorded in the absence of SOD, implicating $O_2^{\cdot-}$ in the mechanism mediating the decrease.

Experiments were performed to examine if NADPH oxidase is the source of the $O_2^{\cdot-}$ implicated in the forskolin-induced pump inhibition. A Na⁺-free superfusate was used. It included the pharmacological NADPH oxidase inhibitor apocynin at a concentration of 10 μ M or it was apocynin free. Experiments were performed with and without 100 nM forskolin included in the superfusate. The Na⁺ concentration in the pipette solution was 10 mM. Results are shown in Figure 6.3. Apocynin had no effect on I_p recorded in the forskolin-free control superfusate. However, it abolished the forskolin-induced decrease in mean I_p

that had been recorded in the absence of apocynin, implicating NADPH oxidase in the mechanism mediating the decrease.

As described in Chapter 5, apocynin is not perfectly selective for NADPH oxidase. Additional experiments were therefore performed using the specific NADPH oxidase inhibitory peptide gp91ds described in Chapter 5. Patch pipette solutions included 10 μ M gp91ds inhibitory peptide. The superfusates contained 100 nM forskolin or they were forskolin-free. Results are included in Figure 6.3. The gp91ds peptide had no effect on I_p recorded in the forskolin-free control superfusate. However, it abolished the forskolin-induced decrease in mean I_p . This confirms the role of NADPH oxidase in the decrease of I_p and implicates ROS/RNS.

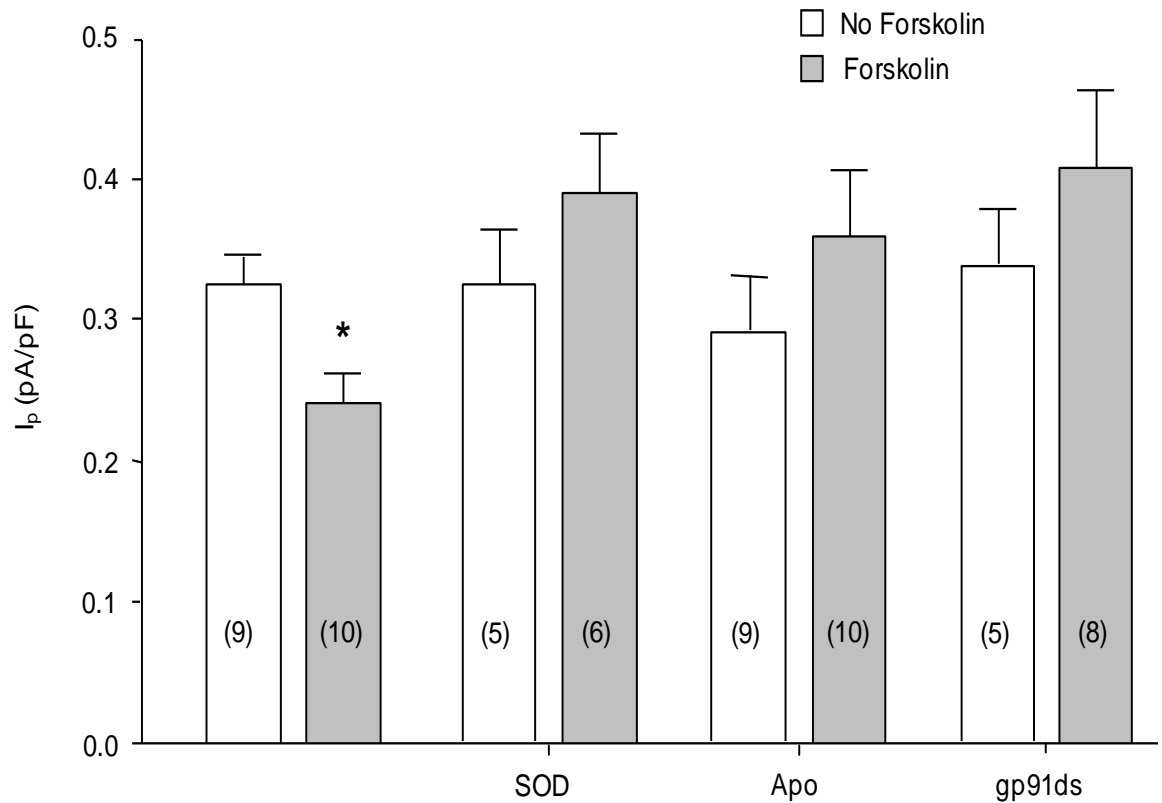


Figure 6.3 Role of superoxide and NADPH oxidase in forskolin-induced $\text{Na}^+\text{-K}^+$ pump inhibition

SOD (200 IU/mL) and the gp91ds peptide (10 μM) were included in the patch pipette solutions as indicated while the readily membrane-permeable apocynin (Apo; 10 μM) was included in the superfusate. Scavenging of $\text{O}_2^{\cdot-}$ or blockade of NADPH oxidase abolished the forskolin-induced $\text{Na}^+\text{-K}^+$ pump inhibition. The pipette Na^+ concentration was 10 mM and the superfusate was Na^+ -free. The test potential was -14 mV. The numbers in parentheses indicate number of experiments. * indicates statistically significant differences ($p < 0.05$).

6.3.3 The role of PKA in forskolin-induced Na⁺-K⁺ pump inhibition

The effect of PKA inhibition on the forskolin-induced decrease in I_p was examined next. Na⁺-free superfusates and pipette solutions that included 10 mM Na⁺ were used. Pipette solutions also included the PKA-inhibitor H-89 in a concentration of 500 nM. The superfusate contained 100 nM forskolin as described above or it was forskolin-free. Results are included in Figure 6.4. H-89 abolished the forskolin-induced decrease in mean I_p .

Phosphorylation of the p47^{phox} subunit of NADPH oxidase is required for activation of oxidase. While PKA can induce limited phosphorylation of p47^{phox}, such phosphorylation does not activate NADPH oxidase. In contrast, PKC induces more extensive phosphorylation of p47^{phox} and activates NADPH oxidase (Park et al., 1997). ϵ PKC-dependent activation of NADPH oxidase was shown to mediate Ang II dependent Na-K pump inhibition. We therefore examined whether PKA-dependent activation of NADPH oxidase may occur via ϵ PKC. Control patch pipette solutions or solutions that included 100 nM PKC ϵ -inhibitory peptide were used. The superfusate contained 100 nM forskolin or it was forskolin-free. Results are included in Figure 6.4. The ϵ PKC-inhibitory peptide abolished the forskolin-induced decrease in mean I_p .

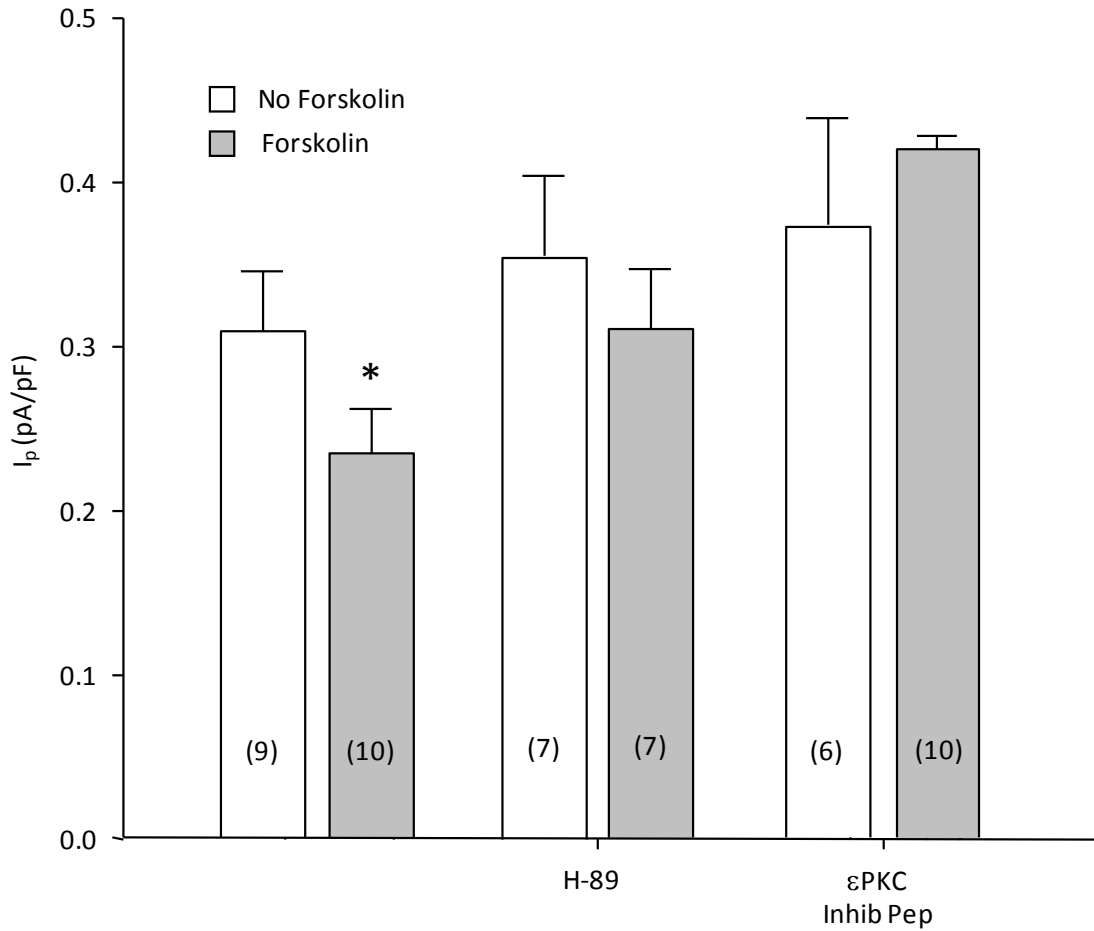


Figure 6.4 Role of PKA and PKC in forskolin-induced $\text{Na}^+\text{-K}^+$ pump inhibition

Patch pipette solutions included 500 nM H-89 or the ϵ PKC-inhibitory peptide (100 nM) as indicated. Inhibition of PKA or ϵ PKC abolished the forskolin-induced pump inhibition. The pipette Na^+ concentration was 10 mM and the superfusate was Na^+ -free. The test potential was -14 mV. The numbers in parentheses indicate number of experiments. * indicates statistically significant differences ($p < 0.05$).

6.4. Discussion.

Data presented in this Chapter shows that activation of adenylyl cyclase by forskolin inhibits the Na⁺-K⁺ pump in cardiac myocytes via an εPKC- and NADPH oxidase- dependent oxidative signalling cascade. The adrenergic control of the Na⁺-K⁺ pump has been difficult to attribute directly to PKA-mediated phosphorylation of the Na⁺-K⁺ pump molecule because its putative phosphorylation sites are poorly accessible to the kinase (Sweadner and Feschenko, 2001). However, PLM has been reported to mediate sarcolemmal Na⁺-K⁺ pump regulation in a study on mouse cardiac myocytes by Despa et al (Despa et al., 2005). The study is difficult to reconcile with results presented in this thesis in terms of the direction of the effect (inhibition vs. stimulation) as well as the molecular mechanism involved. Since the study was published in a high-impact journal and editorialised the discrepancy will be discussed in some detail here. The direction of the response will be discussed first.

6.4.1 Discussion of results in context of previously published data.

The study by Despa et al (2005) demonstrating PKA-dependent stimulation of the Na⁺-K⁺ pump used experimental techniques to study Na⁺-K⁺ pump function identical to those of Han et al (Han et al., 2001) which are critically discussed in Chapter 5. There are some additional technical issues that apply to the study by Despa et al. which are important in the context of the current Chapter. Isolated myocytes were exposed to 1 μM isoproterenol and effects on reported measurements of Na⁺-K⁺ pump activity were attributed to PKA activation. There

was no attempt to substantiate a role of PKA such as using blockers of the β adrenergic receptors coupled to its activation or using compounds that inhibit or activate PKA. This alone makes interpretation difficult. It has, for example, been reported that isoproterenol in the concentration used induces a transient release of nitric oxide in cardiac myocytes (Kanai et al., 1997). Since nitric oxide can induce $\text{Na}^+\text{-K}^+$ pump stimulation in cardiac myocytes (William et al., 2008a, William et al., 2005a), pump stimulation reported by Despa et al cannot be attributed to PKA activation without support from appropriate control experiments.

Experimental recordings assumed to reflect $\text{Na}^+\text{-K}^+$ pump activity also raised serious questions about the validity of the study. Figure 3A in their manuscript shows recordings of membrane currents of a myocytes voltage clamped using patch pipettes with small tips and high resistance that did not allow control of the intracellular Na^+ concentration by the patch pipette solution. Patch clamped myocytes were loaded with Na^+ by exposing them to a K^+ -free superfusate (to inhibit the $\text{Na}^+\text{-K}^+$ pump). They were then re-exposed to K^+ (to activate the pump) and membrane current was measured. A transient outward current was attributed to the $\text{Na}^+\text{-K}^+$ pump and the amplitude of the current was used as an index of activity. The protocol used is a variation of protocols used 20-30 years ago on voltage-clamped multicellular preparations. It was a universal convention to use the rate of decline of current as an index of pump activity, since the amplitude is very susceptible to systematic artefacts arising from changes in ion channel currents with the change in ionic composition of the superfusate used to activate the pump. It is notable that the rate of decay of

current shown in Figure 3A (Despa et al., 2005) is faster under control conditions than after exposure to isoproterenol, *i.e.* indicative of isoproterenol-induced pump *inhibition*. A complicated (but technically flawed –see discussion in Chapter 5) approach using SBFI-fluorescence was used to simultaneously measure intracellular Na⁺ concentration. The rate of decline of intracellular Na⁺ was increased by isoproterenol exposure despite the slower rate of decline of pump current. The opposite effects of isoproterenol on the rate of decline of current attributed to the Na⁺-K⁺ pump and the substrate that fuels the transient increase in pump current, intracellular Na⁺, is difficult to reconcile and casts serious doubt on the validity of the study.

At the molecular level, Despa *et al* attributed the effect of isoproterenol on Na⁺-K⁺ pump function to phosphorylation of the pump-associated FXYD1 protein expressed in heart. However, there was no consideration for how such phosphorylation might alter pump function. If the physical relationship between FXYD1 and the pump's catalytic α subunit is similar to the relationship between FXYD2 and the α subunit in the kidney, it is very difficult to see how phosphorylation of FXYD1 can cause the increase in the pump's Na⁺ affinity that Despa et al. claim. The phosphorylated residues do not seem to be in interaction distance with the domains in the pump molecule where Na⁺ binding occurs (Morth et al., 2007). In addition, any hypothesis implicating phosphorylation of FXYD proteins has the fundamental problem that only FXYD1 has functional phosphorylation sites. FXYD proteins therefore do not provide a generally applicable mechanism for pump regulation, and an alternative mechanism was explored.

Since oxidative signalling mediates Ang II induced pump inhibition (see Chapter 5) and since an increase in adrenergic tone is emerging as a stimulus for oxidant signals, the hypothesis that an adrenergic stimulus regulates pump function was examined in the experiments described in this Chapter (Kou and Michel, 2007, Raju et al., 2005). The cAMP/PKA pathway is known to be coupled to β_1/β_2 adrenergic receptors. Forskolin induced activation of cAMP/PKA pathway resulted in $\text{Na}^+\text{-K}^+$ pump inhibition which was abolished by oxygen radical scavengers or NADPH oxidase inhibitors. The dependence of cAMP-induced pump inhibition on NADPH oxidase is similar to Ang II signalling described in Chapter 5. We therefore examined whether crosstalk existed between these two pathways.

It is well established that Ang II activates NADPH oxidase via PKC. Data presented in Chapter 5 demonstrated that Ang II inhibits the $\text{Na}^+\text{-K}^+$ pump via PKC, NADPH oxidase and superoxide. Although it was expected that the forskolin-induced $\text{Na}^+\text{-K}^+$ pump inhibition described in this Chapter was dependent on activation of PKA, the finding that activation of ϵ PKC was also pivotal in the forskolin-induced pump activation (Figure 6.4) implicated PKA- ϵ PKC crosstalk. While such crosstalk is not widely described, there are three recent reports of similar crosstalk. Cambier et al reported PKA-dependent PKC activation in B-lymphocytes. The specific PKC isoform was not identified (Cambier et al., 1987). Huang et al reported adenosine receptor-mediated, PKA-dependent activation of the atypical ζ PKC isoform in PC12 cells (rat pheochromocytoma cell line)(Huang et al., 2001). However, it is of particular relevance to the data presented here that PKA-dependent activation of the

ϵ PKC isoform has recently been reported (Yao et al., 2008). Dopamine or exposure to ethanol induced activation of ϵ PKC in NG108-15 cells (neurally derived clonal cell line that expresses components of the renin-angiotensin system). Activation was proposed to be due to activation of phospholipase C- β (PLC- β) resulting in an increase in diacylglycerol levels that, in turn, activated ϵ PKC. As was the case in this study, activation was blocked by the specific ϵ PKC inhibitory peptide. The role of PLC in PKA stimulation of PKC, and subsequent activation of NADPH oxidase was supported by inhibitory studies recently performed in our laboratory by my colleagues, and included in a recently published manuscript (White et al., 2010). These results are shown in Figure 6.5. Inhibition of PLC, by U-73122 abolished forskolin-induced pump-inhibition.

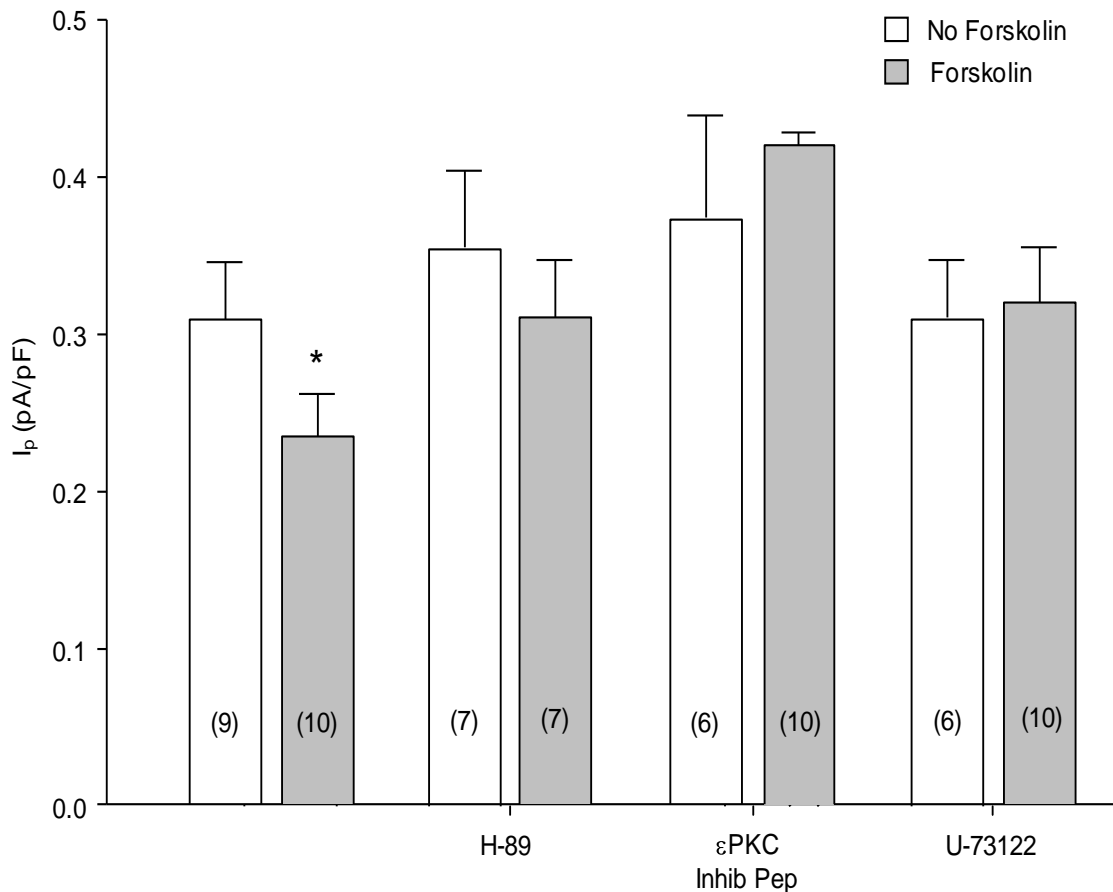


Figure 6.5 Effects of inhibitors of PKA, εPKC and PLC on forskolin-induced Na⁺-K⁺ pump inhibition

Myocytes were perfused with pipette solutions containing H89, εPKC inhibitory peptide or U-73122 (PLC inhibitor) as indicated. The data from myocytes not exposed to inhibitors, previously presented in Figure 6.4 is included for reference. Numbers of myocytes in each group are indicated in parentheses. * indicates a significant difference compared with control.

6.5. Implications

The effect of cAMP-dependent signalling on Na⁺-K⁺ pump function has important implications for our understanding of excitation-contraction coupling in the heart under physiological and pathophysiological conditions. In the normal heart, pump inhibition in combination with an increase in adrenergic drive is expected to increase intracellular Na⁺ and hence Ca²⁺ levels and act in synergy with the well recognized positive inotropic effect of cAMP on Ca²⁺ entry and intracellular Ca²⁺ handling. However, while a modest increase in intracellular Na⁺ levels enhances contractility, the high levels typically seen in heart failure can have the opposite effect and are believed to contribute adversely to its phenotype (Pieske and Houser, 2003, Pieske et al., 2003). It is of particular importance for the present study that adverse effects of NADPH oxidase-dependent redox signalling may also contribute to the development of cardiac failure (Murdoch et al., 2006). Efficacies of treatments that target dysregulation of adrenergic signalling in heart failure trials are consistent with an in vivo relevance of our results. Blockade of adenylyl cyclase-coupled receptors by “β-blockers” may exert some of its benefit (Klein et al., 2003) by reducing oxidative stress, reversing Na⁺-K⁺ pump inhibition and hence reducing cellular Na⁺ overload. This contrasts with the long term detrimental effect of activation of cAMP-dependent signalling by a β₁ adrenergic receptor agonist xameterol (1990) or a phosphodiesterase III inhibitor (Packer et al., 1991) documented in clinical trials. The common role of PKC-dependent NADPH oxidase activation in both Ang II- and cAMP-induced Na⁺-K⁺ pump inhibition may contribute to the well established therapeutic synergy between angiotensin converting enzyme (ACE) inhibitors and β-blockers in heart failure (Klein et al., 2003). Their

combined use is expected to inhibit the shared oxidative signalling pathway more effectively than either group of drugs used alone.

6.6. Summary and Conclusion.

The functional studies described in the results section of this Chapter strongly implicated oxidant signalling in the Na⁺-K⁺ pump inhibition induced by adrenergic activation with cross-over to the same pathway that mediates Ang II-induced oxidant signalling. This represents an entirely new paradigm for adrenergic signalling and regulation of the Na⁺-K⁺ pump in the heart. Subsequent work in the laboratory using both superoxide-sensitive fluorescence and molecular studies of β_1 subunit glutathionylation was performed to independently to complement the functional studies and verify the paradigm shift implicated from the data. The work has established that a downstream oxidative modification of the pump molecule occurs that is identical to that induced by Ang II-dependent oxidant signalling pathways i.e. PKC-dependent activation of NADPH oxidase and subsequent glutathionylation of the β_1 subunit. This data is summarized in Figure 6.6. The implications of this will be discussed in more detail in Chapter 7.

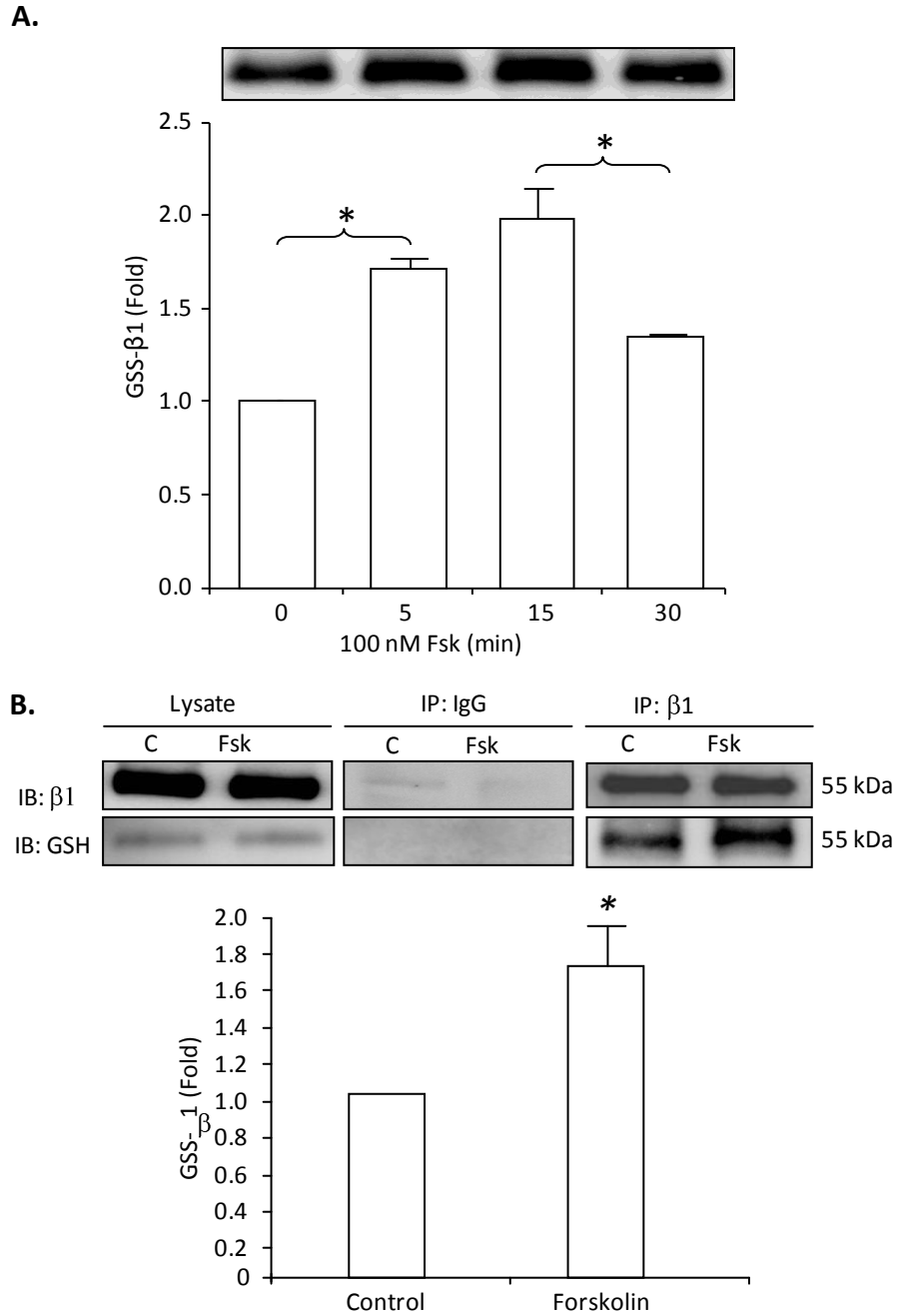


Figure 6.6 Effect of forskolin on glutathionylation of the Na⁺-K⁺ pump's β₁ subunit

A. Immunoblot showing biotinylated GSS-β₁ subunit after exposure of myocytes loaded with biotin-GSH to forskolin for 5, 15 or 30 minutes or vehicle control. B. Effect of forskolin on glutathionylation of β₁ subunit as shown by immunoblotting (IB) β₁ subunit immunoprecipitate (IP) with GSH antibody. The

histograms show mean densitometry of blots from 3-4 experiments, each normalized against control (%). IP indicates the antibody used for immunoprecipitation. IB indicates the antibody used for immunoblot. * indicates significant difference versus control.

\oplus PKA \rightarrow \oplus PLC \rightarrow \oplus ϵ PKC \rightarrow \oplus NADPH Oxidase \rightarrow \ominus Na-K pump

Molecular	H-89 \otimes \uparrow co-IP of PKC/RACK		\uparrow PKC/RACK co-IP	\uparrow p47 ^{phox} /p22 ^{phox} co-IP	\uparrow GSS- β_1 \downarrow α_1/β_1 co-IP
Fluorescence			PKC inhib pep \otimes \uparrow DHE fluorescence	Apocynin and SOD \otimes \uparrow DHE fluorescence	
Functional	H-89 \otimes \downarrow I _p	U73122 \otimes \downarrow I _p	PKC inhib pep \otimes \downarrow I _p	Apocynin and gp91ds \otimes \downarrow I _p	\downarrow I _p

Figure 6.7 Summary of molecular- fluorescence- and patch clamp studies on effects of forskolin

Steps in the signalling pathway that are implicated are shown at the top with stimulation and inhibition indicated by +/- . Blocks of responses to forskolin are indicated below by \times . Glutathionylation of the β_1 subunit is indicated by GSS- β_1 and Na⁺-K⁺ pump current by I_p.(White et al.)(White et al.)(White et al., White et al., 2010)

Chapter 7

Discussion

7.0. Implications and Perspective.

The data presented in Chapter 3 indicate that NO derived from cardiac myocyte NOS stimulates the sarcolemmal Na⁺-K⁺ pump while uncoupling of NOS to produce O₂⁻ induces inhibition as shown in Chapter 4. Such opposing effects would, in principle, allow for hormonal regulation of Na⁺-K⁺ pump activity with appropriate coupling of receptors to sources of NO and ROS/RNS and proximity of Na⁺-K⁺ pump molecules to these sources. In Chapter 5 the well established coupling of Ang II receptors to NAD(P)H oxidase was utilised to test the hypothesis that Ang II induced Na⁺-K⁺ pump inhibition is mediated by O₂⁻ derived from activated NAD(P)H oxidase. This hypothesis was confirmed in a series of functional Na⁺-K⁺ pump studies using highly specific scavengers and peptide inhibitors. Data presented in Chapter 6 demonstrates that activation of the intracellular signalling cascade known to be coupled to β₁/β₂ adrenergic receptors also inhibits the Na⁺-K⁺ pump by sharing a common downstream pathway with Ang II-induced Na⁺-K⁺ pump inhibition. The results are important for our understanding of the basic mechanism of Na⁺-K⁺ pump regulation implying a shift from a traditional emphasis on phosphorylation of the pump molecule or associated FXYD proteins towards a new paradigm with emphasis on oxidative modification. Because heart disease, in particular heart failure, is characterised by dysregulation of NO synthesis, increased oxidative stress in the myocardium and raised myocyte Na⁺ levels (Seddon et al., 2007, Bers et

al., 2003, Despa et al., 2002, Pieske and Houser, 2003, Pieske et al., 2003), as well as elevated neurohormones including Ang II and catecholamine's, the results also have implications for our understanding of pathophysiological processes and therapeutic designs.

7.2. Nitric oxide and the Na⁺-K⁺ pump.

NO is almost the perfect cellular messenger. It is small, readily diffusible, rapidly generated and can also be disposed of quickly. The effects of NO are multiple and complex. It influences many physiological processes, such as neurovascular tone, platelet aggregation, neurotransmission, antimicrobial defence and immunomodulation. Of particular relevance to the work presented here it affects the activity of the isolated Na⁺-K⁺ ATPase in many tissue types including porcine cerebral cortex (Sato et al., 1997), bovine brain, dog kidney (Boldyrev et al., 1997) and hepatic membranes (Muriel P, 2000). Since cellular messenger cascades are not intact in such preparations one has to assume that NO somehow directly interferes with the activity of the ATPase. The addition of a pharmacological donor of NO has been shown to stimulate the Na⁺-K⁺ pump in intact cardiac myocytes previously (William et al., 2005a). In order to examine the physiological significance of this we designed experiments to increase NOS activity within the cell.

The effect of endogenously synthesised NO was examined in the studies presented in Chapter 3 by adding L-arg to the patch pipette solution that perfused the intracellular compartment of isolated voltage clamped myocytes. L-arg induced a highly significant stimulation of I_p. The functional studies

presented in this Thesis were supported with separate work in our laboratory performed by Dr G Figtree. Myocytes were loaded with diacetylated diaminofluorescein 2 (DAF-2DA). This membrane permeable dye is hydrolysed intracellularly by cytosolic esterases releasing DAF-2 which is converted in the presence of NO to DAF-2 triazole (Kojima et al., 1998). The cells were loaded with dye and then examined with laser scanning confocal microscopy. Exposure of cells to L-arg resulted in augmentation of DAF fluorescence, supporting L-arg induced increase in NO synthesis in intact cells as was seen in patched myocytes in Chapter 3. In principle, the L-arg induced $\text{Na}^+\text{-K}^+$ pump stimulation could be due to a direct effect of NO on the pump molecule or the lipid membrane it is embedded in. However, the stimulation was abolished by ODQ, an inhibitor of “soluble” guanylyl cyclase (sGC) that is specifically activated by NO under physiological circumstances. Since sGC mediates synthesis of cGMP a specific downstream cGMP-mediated messenger pathway rather than direct effects of NO or NO derivatives is implicated (Friebe and Koesling, 2003).

The upstream synthesis of NO from L-arg is mediated by NOS. There are 3 known NOS isoforms. NOS1 and NOS3 (the latter also known as “endothelial nitric oxide synthase –eNOS) are constitutively expressed while NOS2 or “iNOS) is induced, usually by pathological stimuli. Subsequent work performed by Dr C Liu in the Laboratory prompted by the results presented in this Thesis has shown that $\text{Na}^+\text{-K}^+$ pump subunits colocalise with eNOS in a caveolin-associated microdomain. The physical proximity is expected to facilitate NO-dependent $\text{Na}^+\text{-K}^+$ pump regulation. This is expected to be important for NOS-coupled hormone receptors since sarcolemmal hormone receptors typically are

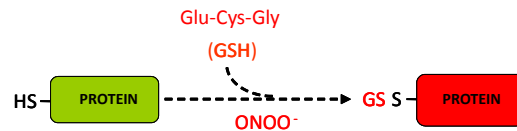
located in membrane caveolae. In agreement with this, natriuretic peptide type C receptors, coupled to NOS, induce stimulation of the Na⁺-K⁺ pump in cardiac myocytes (William et al., 2008a).

7.3. ROS/RNS and the Na⁺-K⁺ pump.

Data presented in Chapters 4, 5 and 6 showed that that uncoupling of NOS by paraquat or activation of NAD(P)H oxidase by Ang II leads to Na⁺-K⁺ pump inhibition that is dependent on O₂⁻. The functional studies presented strongly implicated an oxidative modification of the pump molecule. Oxidative modification of susceptible sulphhydryl groups on proteins may be an important alternative to phosphorylation in regulating their function (Adachi et al., 2004, Clavreul et al., 2006, Cohen and Adachi, 2006). Susceptible sulphhydryl groups may undergo reactions that are essentially irreversible, such as sulphinic or sulphonic oxidation, or, in the presence of the abundant cytosolic tripeptide glutathione, S-glutathionylation may occur. As introduced in Chapter 5 and 6, S-glutathionylation is the formation of a mixed-disulfide bond between a reactive cysteine residue on the protein under regulation, and the tripeptide glutathione. See Figure 7.1.

Protein S-glutathiolation

- S-glutathiolation-



- adduct with –ve charge
- is stable but reversible

Figure 7.1. Stable reversible protein glutathiolation.

S-glutathionation confers a negative charge to a protein molecule that can affect its tertiary structure and cause an alteration in function. This mechanism is reversible. This mechanism is a candidate for indirect coupling of receptor protein kinases and Na⁺-K⁺ pump regulation, as PKC is a well-known activator of NADPH oxidase.

S-glutathionylation is a particularly good candidate for oxidative regulation of function as opposed to more permanent modification because it is reversible and it confers a negative charge which may affect tertiary structure and function in a manner similar to phosphorylation (Cohen and Adachi, 2006). Since the O₂⁻ is derived from PKC-dependent activation of NAD(P)H oxidase (See Chapter 5), S-glutathionylation would also provide a link between receptor-coupled kinase activation and pump regulation. Subsequent studies performed in the laboratory examined if Na⁺-K⁺ pump subunits have “reactive” residues,

i.e. residues that are susceptible to S-glutathionylation. The studies showed that the pump's regulatory β_1 - but not catalytic α_1 subunit was glutathionylated. Results of experiments performed by Dr C Liu are shown in Figure 7.2. There was glutathionylation of the β_1 -subunit in isolated cardiac myocytes at baseline. Glutathionylation was increased with exposure to Ang II (Figtree et al., 2009a). Similar studies to those summarised in Figure 7.2 (also performed by Dr C Liu) demonstrated that exposure of myocytes to paraquat (Figtree et al., 2009a) or forskolin (White et al.) also increased the glutathionylation of the β_1 subunit.

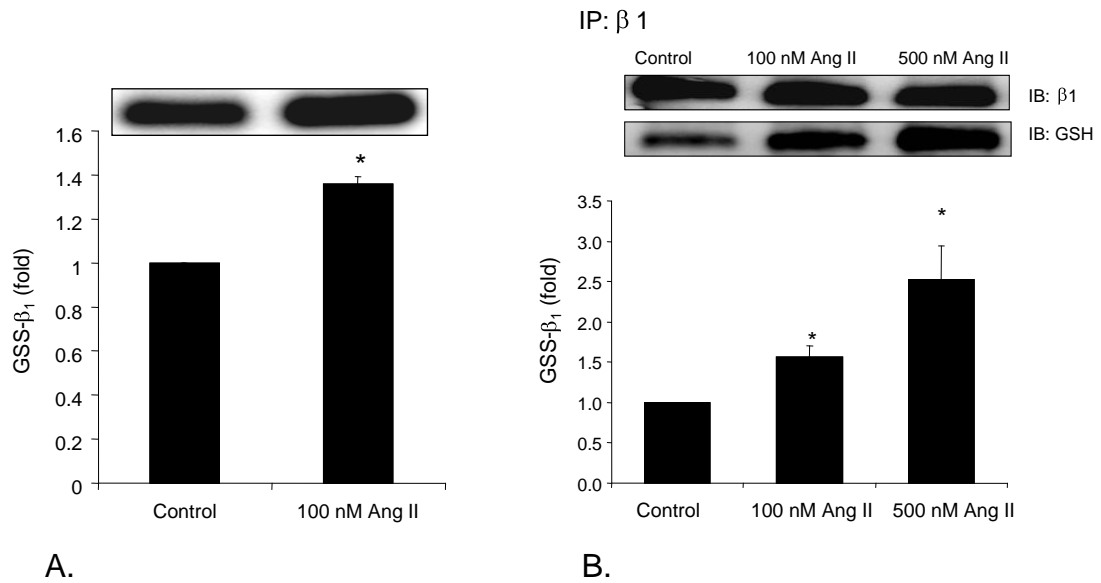


Figure 7.1. Effect of Ang II on glutathionylation of the β_1 subunit (Figtree et al., 2009a).

A. Immunoblots showing biotinylated GSS- β_1 subunit at baseline (control), or after exposure of myocytes to 100 nmol/L (nM) Ang II for 10 minutes. The histogram summarises densitometry of the blots and is standardized to the value of control samples (n=3). B. Ang II induced glutathionylation of β_1 subunit as shown by immunoblotting β_1 subunit immunoprecipitate with GSH antibody. The histogram summarises densitometry of the blots from 3 experiments, and shows a concentration-dependent increase in glutathionylation. *represents significant difference to control.

Glutathionylation of proteins occurs at susceptible cysteine residues, but, intracellularly, the high GSH concentration and reducing environment does not thermodynamically favour glutathionylation of cysteines with typical redox potentials (Gallogly and Mieyal, 2007). Glutathionylation is therefore dependent on subcellular compartmentalization that brings together sources of ROS and target proteins (Martinez-Ruiz and Lamas, 2007). As described previously, the Na⁺-K⁺ pump in cardiac myocytes co-immunoprecipitates with NAD(P)H oxidase, caveolin and with eNOS implying proximity between the Na⁺-K⁺ pump and sources of NO and O₂⁻ in a caveolar microdomain.

NO and O₂⁻ combine to form ONOO⁻ which in turn can oxidize sulphhydryl groups to intermediate forms that promote protein glutathionylation. Glutathione disulphide (GSSG) is an oxidation product of GSH that can mediate glutathionylation of protein cysteine groups by direct disulphide exchange (Gallogly and Mieyal, 2007). However, the local GSH: GSSG ratio would have to shift by several orders of magnitude from the global cell ratio for disulphide exchange to be thermodynamically favoured. GSH can also be oxidized to form S-nitrosyl and sulfenic acid derivatives that are believed to be more likely than GSSG to promote glutathionylation of cysteine residues on proteins in living cells (Gallogly and Mieyal, 2007).

The β₁ subunit has 7 cysteine residues, but 6 are linked in 3 S-S bridges (Shimon et al., 1998), leaving only one candidate for oxidative modification. Studies performed in June 2008 in collaboration with Professor Kaethi Geering from the University of Lausanne, Switzerland have indicated that injection of

ONOO⁻ into oocytes expressing wild-type β_1 subunits with the one remaining free cysteine residue intact induces inhibition of Na⁺-K⁺ pump current. Any effect of ONOO⁻ was eliminated in oocytes expressing a mutant lacking the residue- Cys 45 (Figtree et al., 2009a). These experiments conclusively confirm the identity of the glutathionylated cysteine residue that mediates oxidant induced Na⁺-K⁺ pump inhibition. See Figure 7.3.

Candidate cysteine residue

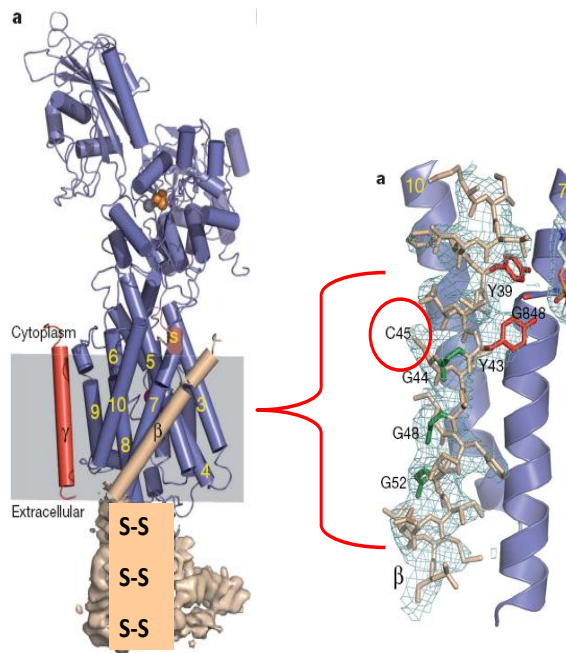


Figure 7.3 Crystal structure of Na⁺-K⁺ pump with Cys45 highlighted.

Mutational studies demonstrated that Cys45 of the b1 subunit is the sole cysteine residue that is glutathionylated, and that its glutathionylation is causally related to oxidative inhibition of the Na⁺-K⁺ pump (modified from (Morth et al., 2007)).

Although accessible in the 3-D structure at the outer face of the helix, the free cysteine residue lies in the transmembrane domain (Morth et al., 2007, Hasler et al., 2001). Within this lipid milieu, the residue would be expected to be poorly accessible to cytosolic, hydrophilic derivatives of GSH. However, its susceptibility to oxidation induced by water soluble Cu-phenanthroline suggests that it nevertheless can be reached by hydrophilic substances, an uncommon feature of membrane-buried cysteine residues in P-type ATPases (Ivanov et al., 2000). In general, oxidation of cysteine residues in transmembrane segments of membrane transporters by water-soluble oxidants implicates proximity of the residues to water-filled cavities (Munson et al., 2000, Tamura et al., 2001). Alternatively, because the acid form of ONOO⁻ is membrane-permeable, the transmembrane cysteine residue of the β_1 subunit may be oxidized to intermediate forms that promote glutathionylation. Analogous to GSH, protein cysteine residues can be oxidized to highly reactive S-nitrosyl or sulphenic acid derivatives (Gallogly and Mieyal, 2007). If such oxidation also induces a change in 3-D structure of the β_1 subunit that allows access of the water soluble GSH, glutathionylation may occur.

Susceptibility to glutathionylation depends on the tertiary structure of a protein and the chemical milieu in the vicinity of cysteines (Ghezzi, 2005).

Glutathionylation as a universal mechanism of Na⁺-K⁺ pump regulation across different species may therefore depend on a high level of homology of the β_1 subunit. A 99% cross-species homology of its transmembrane domain, contrasting to a 57-61% homology to the β_2 and β_3 subunits (Barwe et al., 2007) suggests the transmembrane domain of the β_1 subunit is critical for Na⁺-K⁺ pump regulation and that glutathionylation of the β_1 subunit has a widespread

role in Na⁺-K⁺ pump regulation across species and organs. In contrast, regulation attributed to phosphorylation of FXYP proteins associated with the pump cannot have such broad applicability since only FXYP1 has known functional phosphorylation sites.

The limited homology in the transmembrane domains of the β_1 subunit to the β_2 and β_3 subunits of the Na⁺-K⁺ pump (Barwe et al., 2007) includes the key difference that β_2 and β_3 subunits have no free cysteine residues. The implication is that heterodimer pumps with these subunits cannot be acutely regulated and serve in a constant “housekeeping” role, or, alternatively, that they are regulated by a mechanism other than glutathionylation of the β subunit. Differences in expression of β subunits may give rise to differential regulation of the Na⁺-K⁺ pump, even in subcellular localizations within the same cell. In cardiac myocytes, for example, oxidative signalling may regulate Na⁺-K⁺ pump molecules expressing β_1 subunits in transverse tubules and surface sarcolemmal (Liu and Askari, 2006, Harada et al., 2006), but not pump molecules expressing β_2 subunits at intercalated discs (Harada et al., 2006). A functional effect of glutathionylation is expected to reflect interaction of the β_1 subunit with the catalytic α_1 subunit. Phe 42 and Tyr 43 of the β_1 subunit are within interaction distance of Gly 848 in transmembrane helix 7 of the α_1 subunit ($\alpha M7$). Gly 848 marks a kink in $\alpha M7$ that allows access of the C-terminal of $\alpha M10$ to a pocket between $\alpha M7$, $\alpha M8$ and $\alpha M5$.

Na⁺ binds in this pocket, and the importance of access of the $\alpha M10$ C-terminal to it is indicated by a 26-fold reduction in the Na⁺ affinity when the last five amino acids of $\alpha M10$ are truncated (Morth et al., 2007). Perhaps the access of

the positively charged α M10 C-terminal to the pocket is impaired when the negatively charged glutathione tripeptide binds to Cys 45 in the β_1 subunit near the access route to the pocket. This may impair the effect of the α M10 C-terminal on Na^+ affinity and would be consistent with a reduction in the sensitivity of I_p to intracellular Na^+ induced by oxidant signals (Chapters 3,4,5).

As introduced in Chapter 6, work from this thesis resulted in the investigation of oxidative modification on the kinetics of the $\text{Na}^+\text{-K}^+$ pump. My colleagues have recently demonstrated that glutathionylation decreases the rate of the $E_2 \rightarrow E_1$ conformational change (Figtree et al., 2009b, Winterbourn and Hampton, 2008). The critical role of the β subunit for $\text{Na}^+\text{-K}^+$ ATPase function and ion-binding was recently demonstrated in a high-resolution structure of the shark $\text{Na}^+\text{-K}^+$ ATPase.(Shinoda et al., 2009) It may be of particular importance that Cys45 is only separated from one of the hydrogen bonds linking α M7 with the β_1 subunit by 1 amino acid. The 2 units of the α_1/β_1 heterodimer move relative to each other during the transition from the E_2 to E_1 conformational state described by the Post-Albers scheme, and a charged residue (such as glutathione) on the β_1 subunit may affect this movement and hence functional properties of the enzyme. Exposure of the preparation to ONOO^- inhibited enzymatic activity, and RH421 fluorescence studies indicated that ONOO^- decreased the rate constant for the $E_2 \rightarrow E_1$ conformational change (Figtree et al., 2009a). The shift in the conformational poise toward E_1 may explain the ONOO^- induced increase in apparent affinity to ATP. The $E_2 \rightarrow E_1$ reaction is a good candidate for physiologically significant regulation because, together with regulation by

changes in the intracellular K^+ and Na^+ concentrations, it is the main rate-limiter of the cycle.

7.4. Na^+ - K^+ pump and Heart Failure.

Oxidative modification of the sarcolemmal Na^+ - K^+ pump is likely to be important for the pathophysiology and treatment of heart failure since an increase in cardiac myocyte oxidant stress (Mudd and Kass, 2008) and raised levels of intracellular Na^+ (Pieske and Houser, 2003) are believed to contribute to its clinical manifestations. Oxidative modification of the key export route for Na^+ , the Na^+ - K^+ pump, suggests that oxidant stress and raised myocyte Na^+ are interrelated. However, the pathophysiology of heart failure is complex and there are numerous interrelated factors at play.

The pathophysiology and molecular biology of heart failure are often viewed in terms of abnormalities in *neurohormonal regulation*, intracellular *ion homeostasis*, regulation of *NO synthesis and oxidant stress* or abnormalities in *energy metabolism*. Usually, these apparently diverse concepts are considered in isolation. It is an important implication of the work presented in this thesis that they are likely to be inter-dependent and interact in a manner that may introduce novel targets for therapeutic interventions. Each of these four abnormalities will briefly be discussed in light of work presented in this thesis.

7.4.1 Neurohormonal Regulation

Any decrease in cardiac output, from whatever cause, results in activation of the neurohormonal control axis. Neurohormonal abnormalities include activation of the adrenergic system, the renin-angiotensin-aldosterone system and the hypothalamic-neurohypophyseal system. Activation of the adrenergic system

for a prolonged period, results in an increase in ventricular afterload. This, in turn, puts an increased strain on the compromised left ventricle. Sustained long-term activation of the adrenergic system can result in hypertrophy, myocardial damage, arrhythmias, and possibly myocardial calcium overload and apoptosis (Anversa et al., 1998). Activation of the renin-angiotensin-aldosterone system results in increased production of Ang II. Sustained elevations of Ang II can cause an increase in ventricular afterload and myocardial hypertrophy. In the longer-term the increased hormone level can lead to apoptosis, interstitial fibrosis and increased secretion of aldosterone (Swedberg et al., 1990). These changes within the myocardium increase passive stiffness of the ventricles and vascular bed, interfere with ventricular filling, and reduce arterial compliance. This often leads to ventricular remodelling which is a maladaptive response and in the long term is not of benefit to the patient. The neurohormonal hypothesis has been very successful in developing and/or explaining beneficial therapeutic interventions with angiotensin converting enzyme inhibitors (ACE-I), angiotensin receptor blockers (ARBs), aldosterone antagonists and adrenergic β receptor blockers. As explored in this work, both Ang II, and forskolin (mimicking activation of β_1/β_2 adrenergic receptors) leads to inhibition of the cardiac $\text{Na}^+\text{-K}^+$ pump.

7.4.2 Ionic hypothesis

In congestive cardiac failure, the hypothesis that the “electro-mechanical phenotype” of abnormalities in cardiac contractility and frequent cardiac arrhythmias in heart failure are as a result of abnormalities in myocytes ion

homeostasis has mostly been focussed on the cellular handling of Ca^{2+} (Houser and Lakatta, 1999). Much work has been done on storage of Ca^{2+} in the sarcoplasmic reticulum and the uptake of Ca^{2+} into it, mediated by a Ca^{2+} ATPase. Other studies have been focussed on the cellular export of Ca^{2+} across the sarcolemmal, mostly mediated by the Na^+ - Ca^{2+} exchanger. The main consistent pattern that has emerged is that an increase in total cell Ca^{2+} is a key feature in electro-mechanical dissociation. Since the intracellular Na^+ concentration $[\text{Na}^+]_i$ in the cardiac myocyte is the key determinant of intracellular Ca^{2+} due to the operation of the Na^+ - Ca^{2+} exchanger attention has been drawn to $[\text{Na}^+]_i$ in the failing heart. It has become firmly established that $[\text{Na}^+]_i$ is elevated in heart failure and that this plays a key role in its pathophysiology in both humans and in animal models of heart failure (Pieske et al., 2002, Pieske and Houser, 2003, Pieske et al., 2003, Despa et al., 2002) (See Chapter 1). This increased $[\text{Na}^+]_i$ reduces cellular export of Ca^{2+} via Na^+ - Ca^{2+} exchange and hence results in an increase in total cell Ca^{2+} .

7.4.3 Role of ROS /RNS in Heart Failure

The dysregulation of the NOS pathway and the production of peroxynitrite may have an important role in the development of heart failure. The amount of available NO is variable and depends on the availability of both substrate and co-factors as discussed in Chapters 3 and 4. The lack of either of these two important elements leads to the generation of oxygen radicals such as peroxynitrite (ONOO^-). As discussed ONOO^- can significantly alter glutathionation, nitration and oxidative reactions. The amount of available NO is

important as it has both paracrine and autocrine functions on the cardiac myocyte. At low doses NO influences electro-mechanical coupling and has a positive inotropic effect (Mohan et al., 1996). At higher doses it augments diastolic function and myocyte relaxation (Mohan et al., 1996, Shah and MacCarthy, 2000, Vila-Petroff et al., 1999). In heart failure the increase in oxygen radicals may have many sources including xanthine oxidase (Ekelund et al., 1999), mitochondria (Ide et al., 1999) and NAD(P)H oxidase as discussed in Chapter 5. The increase in the oxygen radical species and reduction in NO leads to a reduction in the I_p of the $Na^+ - K^+$ pump as demonstrated in Chapters 4 and 5. The reduction of I_p of the $Na^+ - K^+$ pump and therefore by implication an increase in the intracellular Na^+ , leads to an alteration in Ca^{2+} handling and electrolyte imbalances. This links the neurohormonal theory and the electrolyte imbalance theory. The regulatory hormones such as Ang II cause an increase in the ROS/RNS which, as demonstrated, reduce the I_p . These electrolyte imbalances cause a reduction in myocyte function and lead to the clinical manifestations of congestive cardiac failure.

Takosubo cardiomyopathy is a transient cardiac condition due to apical ballooning of the left ventricle. It often mimics an acute coronary syndrome. This leads to a marked reduction in left ventricular function. It was first described in Japan in 1990 by Sato. (Sato et al., 1997). The syndrome appears to be triggered by significant emotional or physical exertion. The etiology is not known however a common mechanism is the release of catecholamines and stunning of the myocardium. This hypothesis would fit with the findings of this thesis as discussed in the previous chapters. The excessive release of catecholamine's

causes the generation of ROS/RNS which leads reduced myocardial contraction.

7.4.4 Energy Starvation

Heart failure has also often been viewed as a problem primarily related to cardiac energy metabolism. The heart is an avaricious consumer of free fatty acids (FFA) and glucose. FFA metabolism is the main source of energy of the myocardium in the non- stressed heart. The myocardium uses about 6 kg of ATP daily (Neubauer, 2007). The myocardium must provide a steady stream of ATP to prevent failure of contraction of the myofibrils. Heart failure has also often been viewed as a problem primarily related to cardiac energy metabolism. In a stressed state, such as advanced heart failure, the myocardium switches to carbohydrate metabolism as a source of energy. While initially adaptive it quickly becomes harmful as there is an increase in insulin resistance and loss of metabolic plasticity.

Much early work was focussed on cellular levels of high-energy phosphates, including ATP and creatine phosphate. The cellular level of ATP is maintained until the end stages of advanced HF. The transport of substrates in to the abundant mitochondria is vital to sustain adequate amounts of ATP from phosphocreatine and ADP. There are a number of transporters that are Na⁺ dependent, including the GLUT transporters (which were previously discussed in Chapter 1) and the Na⁺ dependant creatine co-transporters. Creatine is produced by both the liver and the kidneys. It is transported across the cardiac

membrane against a 50 fold gradient by the Na^+ dependant creatine co-transporter (Neubauer, 2007). Thus any change in the Na^+ level will impact on the transporters which play a vital role in the chain of energy delivery and utilisation within the myocyte. This interruption of the substrate delivery may have a downstream effect on the electro mechanical coupling of the myocardium which is ATP dependant.

7.5 Unifying Scheme and implications for future work

The work presented in this thesis has identified a link between ROS/RNS and signalling mediated by neurohormonal activation in the control of cardiac myocyte Na^+ , and, by inference Ca^{2+} . This indicates that abnormalities in ROS/RNS, intracellular ions and neurohormones should not be seen isolation when considering the pathophysiology of heart failure. Because of the dependence of cardiac myocyte energy metabolism on the transmembrane gradient for Na^+ it is also reasonable to think that myocardial energy starvation in heart failure is readily integrated in an overall inter-dependence with neurohormonal abnormalities and dysregulation of intracellular ions and myocardial ROS/RNS in the pathogenesis of heart failure. Clinical trials of antioxidants in cardiac failure have been disappointing. There are many reasons for this. The antioxidants used in the trials such as vitamin C, vitamin E and co-enzyme Q10 complex are non-specific inhibitors. They also have a low reaction rate with ROS/RNS or are non-catalytic antioxidants. The dose used was also inadequate. Tackling an increase in ROS/RNS either needs a specific target downstream to inhibit or to be an antioxidant with a high reaction rate.

Allopurinol, a XOR inhibitor reduces the level of ROS/RNS in endothelium and has been shown to increase NO stimulated blood flow in both smokers and diabetics experimentally (Guthikonda et al., 2003, Butler et al., 2000). There may be a possible role for its use in heart failure as Cappola et al showed the infusion of allopurinol into the coronary vasculature reduced myocardial O₂ consumption (Cappola et al., 2001). There may be a role for use of antioxidants in the treatment of heart failure however, at this time they are too non-specific in their functions and require further experimental work and drug development. The success of receptor-based therapies vs. the lack of efficacy of non-specific antioxidant approaches (Mudd and Kass, 2008) may be an indication of the pivotal importance of cellular microdomains in the regulation of normal and abnormal cell function. One cardiomyopathy that may be significant in the context of this thesis finding is Takotsubo cardiomyopathy. Takotsubo cardiomyopathy is a transient cardiac condition first described by Sato in 1990 (Sato et al.). It is a condition that causes marked apical hypokinesis of the left ventricle and a reduction in ventricular function. It appears to be triggered by significant emotional stress. The etiology is not known but it is thought to be linked to a large sudden burst of catecholamine release. 95% of cases recover within 6-8 weeks. The release of noradrenaline is hypothesised causes a significant increase in the amount of ROS/RNS generated which leads to myocardial dysfunction as outlined in the previous chapters of this thesis. The findings presented in this thesis that neurohormonally induced oxidative signalling inhibits the Na⁺-K⁺ pump via PKC dependent activation of NAD(P)H oxidase have led to the identification of a specific oxidative modification of the β₁ subunit of the Na⁺-K⁺ pump that mediates this. These combined findings

provide novel therapeutic targets that, if successfully interrupted may improve cellular Na^+ - and Ca^{2+} handling under conditions of dysregulated neurohormones and elevated cellular oxidative stress. This is the ongoing focus of the Laboratory.

8.0 Bibliography

- (1990) Xamoterol in severe heart failure. The Xamoterol in Severe Heart Failure Study Group. *Lancet*, 336, 1-6.
- ABEL, E. D. (2004) Glucose transport in the heart. *Front Biosci*, 9, 201-15.
- ADACHI, T., WEISBROD, R. M., PIMENTEL, D. R., YING, J., SHAROV, V. S., SCHONEICH, C. & COHEN, R. A. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med*, 10, 1200-7.
- ALDERTON WK, COOPER CE & KNOWLES RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J*, 357, 593-615.
- ANVERSA, P., LERI, A., BELTRAMI, C. A., GUERRA, S. & KAJSTURA, J. (1998) Myocyte death and growth in the failing heart. *Lab Invest*, 78, 767-86.
- APELL, H. J. & KARLISH, S. J. (2001) Functional properties of Na,K-ATPase, and their structural implications, as detected with biophysical techniques. *J Membr Biol*, 180, 1-9.
- BABIOR, B. M. (2000) The NADPH oxidase of endothelial cells. *IUBMB Life*, 50, 267-9.
- BAHINSKI, A., NAIRN, A. C., GREENGARD, P. & GADSBY, D. C. (1989) Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*, 340, 718-21.
- BALLIGAND, J. L. (2000) [Beta 3 adrenergic receptor: physiologic role and potential therapeutic applications]. *Bull Mem Acad R Med Belg*, 155, 311-7; discussion 317-9.
- BALLIGAND, J. L., UNGUREANU-LONGROIS, D., SIMMONS, W. W., PIMENTAL, D., MALINSKI, T. A., KAPTURCZAK, M., TAHA, Z., LOWENSTEIN, C. J., DAVIDOFF, A. J., KELLY, R. A. & ET AL. (1994) Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem*, 269, 27580-8.
- BAROUCH, L. A., HARRISON, R. W., SKAF, M. W., ROSAS, G. O., CAPPOLA, T. P., KOBEISSI, Z. A., HOBAI, I. A., LEMMON, C. A., BURNETT, A. L., O'ROURKE, B., RODRIGUEZ, E. R., HUANG, P. L., LIMA, J. A., BERKOWITZ, D. E. & HARE, J. M. (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature*, 416, 337-9.
- BARWE, S. P., KIM, S., RAJASEKARAN, S. A., BOWIE, J. U. & RAJASEKARAN, A. K. (2007) Janus model of the Na,K-ATPase beta-subunit transmembrane domain: distinct faces mediate alpha/beta assembly and beta-beta homo-oligomerization. *J Mol Biol*, 365, 706-14.

- BEDARD, K. & KRAUSE, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*, 87, 245-313.
- BERK, B. C., ELDER, E. & MITSUKA, M. (1990) Hypertrophy and hyperplasia cause differing effects on vascular smooth muscle cell Na⁺/H⁺ exchange and intracellular pH. *J Biol Chem*, 265, 19632-7.
- BERS, D. M. (2001) *Excitation-Contraction Coupling and Cardiac Contractile Force* Dordrecht, The Netherlands, Kluwer Academic Press:427.
- BERS, D. M., BARRY, W. H. & DESPA, S. (2003) Intracellular Na⁺ regulation in cardiac myocytes. *Cardiovasc Res*, 57, 897-912.
- BIBERT, S., ROY, S., SCHAER, D., HORISBERGER, J. D. & GEERING, K. (2008) Phosphorylation of phospholemman (FXD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *J Biol Chem*, 283, 476-86.
- BLANCO, G., SANCHEZ, G., MELTON, R. J., TOURTELLOTTE, W. G. & MERCER, R. W. (2000) The alpha4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *J Histochem Cytochem*, 48, 1023-32.
- BOLDYREV, A. A., BULYGINA, E. R., KRAMARENKO, G. G. & VANIN, A. F. (1997) Effect of nitroso compounds on Na/K-ATPase. *Biochim Biophys Acta*, 1321, 243-51.
- BORGES, V. C., ROCHA, J. B. & NOGUEIRA, C. W. (2005) Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. *Toxicology*, 215, 191-7.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001a) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na(+)-K(+) pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001b) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na⁺-K⁺ pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., GRAY, D. F., MIHAILIDOU, A. S. & RASMUSSEN, H. H. (1999) Angiotensin regulates the selectivity of the Na⁺-K⁺ pump for intracellular Na⁺. *Am J Physiol*, 277, C461-8.
- BUHAGIAR, K. A., HANSEN, P. S., KONG, B. Y., CLARKE, R. J., FERNANDES, C. & RASMUSSEN, H. H. (2004) Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes. *Am J Physiol Cell Physiol*, 286, C398-405.
- BUNDGAARD H, G. A., HAMILTON EJ, WHITE CN, RASMUSSEN HH (2006) Beta-3 receptors mediate adrenergic stimulation of the sarcolemmal Na⁺-K⁺ pump. *Biophys J suppl*, 556a.
- BUTLER, R., MORRIS, A. D., BELCH, J. J., HILL, A. & STRUTHERS, A. D. (2000) Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension*, 35, 746-51.
- CAI, H., GRIENDLING, K. K. & HARRISON, D. G. (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*, 24, 471-8.
- CAI, H. & HARRISON, D. G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*, 87, 840-4.

- CAMBIER, J. C., NEWELL, M. K., JUSTEMENT, L. B., MCGUIRE, J. C., LEACH, K. L. & CHEN, Z. Z. (1987) Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature*, 327, 629-32.
- CAPPOLA, T. P., KASS, D. A., NELSON, G. S., BERGER, R. D., ROSAS, G. O., KOBEISSI, Z. A., MARBAN, E. & HARE, J. M. (2001) Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation*, 104, 2407-11.
- CHARPENTIER, F., LEGATO, M. J., STEINBERG, S. F., COHEN, I. S. & ROSEN, M. R. (1996) Beta-adrenergic modulation of Na-K pump activity in young and adult canine cardiac Purkinje fibers. *Am J Physiol*, 271, H706-12.
- CHENG, X. J., HOOG, J. O., NAIRN, A. C., GREENGARD, P. & APERIA, A. (1997) Regulation of rat Na(+)-K(+)-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am J Physiol*, 273, C1981-6.
- CLAUSEN, T., VAN HARDEVELD, C. & EVERTS, M. E. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev*, 71, 733-74.
- CLAVREUL, N., BACHSCHMID, M. M., HOU, X., SHI, C., IDRIZOVIC, A., IDO, Y., PIMENTEL, D. & COHEN, R. A. (2006) S-glutathiolation of p21ras by peroxynitrite mediates endothelial insulin resistance caused by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol*, 26, 2454-61.
- CLOSS, E. I., SCHELD, J. S., SHARAFI, M. & FORSTERMANN, U. (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol*, 57, 68-74.
- COHEN, R. A. & ADACHI, T. (2006) Nitric-oxide-induced vasodilatation: regulation by physiologic s-glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc Med*, 16, 109-14.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003a) Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci*, 18, 119-24.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003b) Themes in ion pump regulation. *Ann N Y Acad Sci*, 986, 579-86.
- CORNELIUS, F., MAHMMOUD, Y. A. & CHRISTENSEN, H. R. (2001) Modulation of Na,K-ATPase by associated small transmembrane regulatory proteins and by lipids. *J Bioenerg Biomembr*, 33, 415-23.
- DAIBER, A., ZOU, M. H., BACHSCHMID, M. & ULLRICH, V. (2000) Ebselen as a peroxynitrite scavenger in vitro and ex vivo. *Biochem Pharmacol*, 59, 153-60.
- DE GASPARO, M. & SIRAGY, H. M. (1999) The AT2 receptor: fact, fancy and fantasy. *Regul Pept*, 81, 11-24.
- DESPA, S., BOSSUYT, J., HAN, F., GINSBURG, K. S., JIA, L. G., KUTCHAI, H., TUCKER, A. L. & BERS, D. M. (2005) Phospholemman-phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. *Circ Res*, 97, 252-9.

- DESPA, S., ISLAM, M. A., WEBER, C. R., POGWIZD, S. M. & BERS, D. M. (2002) Intracellular Na(+) concentration is elevated in heart failure but Na/K pump function is unchanged. *Circulation*, 105, 2543-8.
- DOEGE, H., BOCIANSKI, A., SCHEEPERS, A., AXER, H., ECKEL, J., JOOST, H. G. & SCHURMANN, A. (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J*, 359, 443-9.
- DUDZINSKI, D. M., IGARASHI, J., GREIF, D. & MICHEL, T. (2006) The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol*, 46, 235-76.
- EKELUND, U. E., HARRISON, R. W., SHOKEK, O., THAKKAR, R. N., TUNIN, R. S., SENZAKI, H., KASS, D. A., MARBAN, E. & HARE, J. M. (1999) Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circ Res*, 85, 437-45.
- ELLIS, D. Z., RABE, J. & SWEADNER, K. J. (2003) Global loss of Na,K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci*, 23, 43-51.
- ERLENKAMP, S., GLITSCH, H. G. & KOCKSKAMPER, J. (2002) Dual regulation of cardiac Na⁺-K⁺ pumps and CFTR Cl⁻ channels by protein kinases A and C. *Pflugers Arch*, 444, 251-62.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009a) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009b) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FLIEGEL, L. (2008) Molecular biology of the myocardial Na⁺/H⁺ exchanger. *J Mol Cell Cardiol*, 44, 228-37.
- FLIEGEL, L. & FROHLICH, O. (1993) The Na⁺/H⁺ exchanger: an update on structure, regulation and cardiac physiology. *Biochem J*, 296 (Pt 2), 273-85.
- FOLLMER, C. H., TEN EICK, R. E. & YEH, J. Z. (1987) Sodium current kinetics in cat atrial myocytes. *J Physiol*, 384, 169-97.
- FORBUSH, B., 3RD, KAPLAN, J. H. & HOFFMAN, J. F. (1978) Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry*, 17, 3667-76.
- FRIEBE, A. & KOESLING, D. (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res*, 93, 96-105.
- FULLER, W., EATON, P., BELL, J. R. & SHATTOCK, M. J. (2004) Ischemia-induced phosphorylation of phospholemman directly activates rat cardiac Na/K-ATPase. *Faseb J*, 18, 197-9.
- FULLER, W., HOWIE, J., MCLATCHIE, L. M., WEBER, R. J., HASTIE, C. J., BURNES, K., PAVLOVIC, D. & SHATTOCK, M. J. (2009) FXD1 phosphorylation in vitro and in adult rat cardiac myocytes: threonine 69 is

- a novel substrate for protein kinase C. *Am J Physiol Cell Physiol*, 296, C1346-55.
- GADSBY, D. C. (2007) Structural biology: ion pumps made crystal clear. *Nature*, 450, 957-9.
- GADSBY, D. C., KIMURA, J. & NOMA, A. (1985) Voltage dependence of Na/K pump current in isolated heart cells. *Nature*, 315, 63-5.
- GADSBY, D. C. & NAKAO, M. (1989) Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 511-37.
- GADSBY, D. C., NAKAO, M. & BAHINSKI, A. (1989) Voltage dependence of transient and steady-state Na/K pump currents in myocytes. *Mol Cell Biochem*, 89, 141-6.
- GALLOGLY, M. M. & MIEYAL, J. J. (2007) Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr Opin Pharmacol*, 7, 381-91.
- GAO, J., COHEN, I. S., MATHIAS, R. T. & BALDO, G. J. (1998) The inhibitory effect of beta-stimulation on the Na/K pump current in guinea pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. *Pflugers Arch*, 435, 479-84.
- GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1992) Isoprenaline, Ca²⁺ and the Na(+)-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 449, 689-704.
- GAO, J., MATHIAS, R. T., COHEN, I. S., WANG, Y., SUN, X. & BALDO, G. J. (1999) Activation of PKC increases Na⁺-K⁺ pump current in ventricular myocytes from guinea pig heart. *Pflugers Arch*, 437, 643-51.
- GARCIA, A., BUNDGAARD, H., HAMILTON, E., LIU, C., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) beta-3 adrenergic stimulation of the Na⁺-K⁺ pump is mediated by NO-dependent deglutathionylation of the beta-1 pump subunit. *12th International ATPase Conference*. Aarhus.
- GARTY, H. & KARLISH, S. J. (2006) Role of FXYD proteins in ion transport. *Annu Rev Physiol*, 68, 431-59.
- GEERING, K. (2001) The functional role of beta subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr*, 33, 425-38.
- GHEZZI, P. (2005) Regulation of protein function by glutathionylation. *Free Radic Res*, 39, 573-80.
- GLITSCH, H. G. (2001) Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev*, 81, 1791-826.
- GOLIGORSKY, M. S., CHEN, J. & BRODSKY, S. (2001) Workshop: endothelial cell dysfunction leading to diabetic nephropathy : focus on nitric oxide. *Hypertension*, 37, 744-8.
- GOLIGORSKY, M. S. & GROSS, S. S. (2001) The ins and outs of endothelial dysfunction: much ado about NO-thing. *Drug News Perspect*, 14, 133-42.
- GRIENGLING, K. K., MINIERI, C. A., OLLERENSHAW, J. D. & ALEXANDER, R. W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*, 74, 1141-8.
- GRIENGLING, K. K., SORESCU, D. & USHIO-FUKAI, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*, 86, 494-501.

- GUIMBAL, C. & KILIMANN, M. W. (1993) A Na(+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem*, 268, 8418-21.
- GUTHIKONDA, S., SINKEY, C., BARENZ, T. & HAYNES, W. G. (2003) Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers. *Circulation*, 107, 416-21.
- HAN, F., BOSSUYT, J., DESPA, S., TUCKER, A. L. & BERS, D. M. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na⁺/K⁺ pump function in cardiac myocytes. *Circ Res*, 99, 1376-83.
- HAN, J., KIM, N., KIM, E., HO, W. K. & EARM, Y. E. (2001) Modulation of ATP-sensitive potassium channels by cGMP-dependent protein kinase in rabbit ventricular myocytes. *J Biol Chem*, 276, 22140-7.
- HANSEN, P. S., BUHAGIAR, K. A., KONG, B. Y., CLARKE, R. J., GRAY, D. F. & RASMUSSEN, H. H. (2002) Dependence of Na⁺-K⁺ pump current-voltage relationship on intracellular Na⁺, K⁺, and Cs⁺ in rabbit cardiac myocytes. *Am J Physiol Cell Physiol*, 283, C1511-21.
- HANSEN, P. S., CLARKE, R. J., BUHAGIAR, K. A., HAMILTON, E., GARCIA, A., WHITE, C. & RASMUSSEN, H. H. (2007) Alloxan-induced diabetes reduces sarcolemmal Na⁺-K⁺ pump function in rabbit ventricular myocytes. *Am J Physiol Cell Physiol*, 292, C1070-1077.
- HARADA, K., LIN, H., ENDO, Y., FUJISHIRO, N., SAKAMOTO, Y. & INOUE, M. (2006) Subunit composition and role of Na⁺,K⁺-ATPases in ventricular myocytes. *J Physiol Sci*, 56, 113-21.
- HARRISON, D. G. (1997) Endothelial function and oxidant stress. *Clin Cardiol*, 20, 11-17.
- HASLER, U., CRAMBERT, G., HORISBERGER, J. D. & GEERING, K. (2001) Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. *J Biol Chem*, 276, 16356-64.
- HELIN, P. & LORENZEN, I. (1976) Seasonal variations in the susceptibility of the aortic wall to atherosclerosis. Biochemical studies of glycosaminoglycans and collagen of rabbit atherosclerosis. *Atherosclerosis*, 24, 259-66.
- HERRERA, V. L., EMANUEL, J. R., RUIZ-OPAZO, N., LEVENSON, R. & NADAL-GINARD, B. (1987) Three differentially expressed Na,K-ATPase alpha subunit isoforms: structural and functional implications. *J Cell Biol*, 105, 1855-65.
- HESS, D. T., MATSUMOTO, A., KIM, S. O., MARSHALL, H. E. & STAMLER, J. S. (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*, 6, 150-66.
- HEVEL, J. M. & MARLETTA, M. A. (1992) Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry*, 31, 7160-5.
- HINGTGEN, S. D., TIAN, X., YANG, J., DUNLAY, S. M., PEEK, A. S., WU, Y., SHARMA, R. V., ENGELHARDT, J. F. & DAVISSON, R. L. (2006) Nox2-containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. *Physiol Genomics*, 26, 180-91.

- HOFFMANN, C., LEITZ, M. R., OBERDORF-MAASS, S., LOHSE, M. J. & KLOTZ, K. N. (2004) Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*, 369, 151-9.
- HOOL LC, GRAY DF, ROBINSON BG & RASMUSSEN HH (1996) Angiotensin-converting enzyme inhibitors regulate the Na(+)-K+ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-C180.
- HOOL, L. C., GRAY, D. F., ROBINSON, B. G. & RASMUSSEN, H. H. (1996) Angiotensin-converting enzyme inhibitors regulate the Na⁺-K⁺ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-80.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995a) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na(+)-K+ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995b) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na⁺-K⁺ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOUSER, S. R. & LAKATTA, E. G. (1999) Function of the cardiac myocyte in the conundrum of end-stage, dilated human heart failure. *Circulation*, 99, 600-4.
- HUANG, B., BLANCO, G., MERCER, R. W., FLEMING, T. & PEPOSE, J. S. (2003) Human corneal endothelial cell expression of Na⁺,K⁺-adenosine triphosphatase isoforms. *Arch Ophthalmol*, 121, 840-5.
- HUANG, N. K., LIN, Y. W., HUANG, C. L., MESSING, R. O. & CHERN, Y. (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. *J Biol Chem*, 276, 13838-46.
- IDE, T., TSUTSUI, H., KINUGAWA, S., UTSUMI, H., KANG, D., HATTORI, N., UCHIDA, K., ARIMURA, K., EGASHIRA, K. & TAKESHITA, A. (1999) Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res*, 85, 357-63.
- IKEHARA, T., YAMAGUCHI, H., HOSOKAWA, K., TAKAHASHI, A. & MIYAMOTO, H. (1993) Kinetic study on the effects of intracellular K⁺ and Na⁺ on Na⁺, K⁺, Cl⁻ cotransport of HeLa cells by Rb⁺ influx determination. *J Membr Biol*, 132, 115-24.
- IVANOV, A., ZHAO, H. & MODYANOV, N. N. (2000) Packing of the transmembrane helices of Na,K-ATPase: direct contact between beta-subunit and H8 segment of alpha-subunit revealed by oxidative cross-linking. *Biochemistry*, 39, 9778-85.
- JIA, L. G., DONNET, C., BOGAEV, R. C., BLATT, R. J., MCKINNEY, C. E., DAY, K. H., BERR, S. S., JONES, L. R., MOORMAN, J. R., SWEADNER, K. J. & TUCKER, A. L. (2005) Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice. *Am J Physiol Heart Circ Physiol*, 288, H1982-8.
- JOHNSON, F. & GIULIVI, C. (2005) Superoxide dismutases and their impact upon human health. *Mol Aspects Med*, 26, 340-52.
- KANAI, A. J., MESAROS, S., FINKEL, M. S., ODDIS, C. V., BIRDER, L. A. & MALINSKI, T. (1997) Beta-adrenergic regulation of constitutive nitric oxide synthase in cardiac myocytes. *Am J Physiol*, 273, C1371-7.

- KARLISH, S. J., YATES, D. W. & GLYNN, I. M. (1978) Elementary steps of the (Na⁺ + K⁺)-ATPase mechanism, studied with formycin nucleotides. *Biochim Biophys Acta*, 525, 230-51.
- KLEIN, L., O'CONNOR, C. M., GATTIS, W. A., ZAMPINO, M., DE LUCA, L., VITARELLI, A., FEDELE, F. & GHEORGHIADU, M. (2003) Pharmacologic therapy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations. *Am J Cardiol*, 91, 18F-40F.
- KOCKSKAMPER, J., ERLINKAMP, S. & GLITSCH, H. G. (2000) Activation of the cAMP-protein kinase A pathway facilitates Na⁺ translocation by the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 523 Pt 3, 561-74.
- KOCKSKAMPER, J., SENDHOFF, K., ERLINKAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOCKSKÄMPER, J., SENDHOFF, K., ERLINKAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOJIMA, H., NAKATSUBO, N., KIKUCHI, K., KAWAHARA, S., KIRINO, Y., NAGOSHI, H., HIRATA, Y. & NAGANO, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem*, 70, 2446-53.
- KONG, B. Y. & CLARKE, R. J. (2004) Identification of potential regulatory sites of the Na⁺,K⁺-ATPase by kinetic analysis. *Biochemistry*, 43, 2241-50.
- KOU, R. & MICHEL, T. (2007) Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*, 282, 32719-29.
- KURZ, S. & HARRISON, D. G. (1997) Insulin and the arginine paradox. *J Clin Invest*, 99, 369-70.
- LAMBETH, J. D., KAWAHARA, T. & DIEBOLD, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med*, 43, 319-31.
- LASS, A., SUESSENBACHER, A., WOLKART, G., MAYER, B. & BRUNNER, F. (2002) Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol*, 61, 1081-8.
- LASTRA, G. & MANRIQUE, C. (2007) The expanding role of oxidative stress, renin angiotensin system, and beta-cell dysfunction in the cardiometabolic syndrome and Type 2 diabetes mellitus. *Antioxid Redox Signal*, 9, 943-54.
- LAUGER, P. (1991) Kinetic basis of voltage dependence of the Na,K-pump. *Soc Gen Physiol Ser*, 46, 303-15.
- LEMAY, J., HOU, Y. & DEBLOIS, D. (2000) Evidence that nitric oxide regulates AT1-receptor agonist and antagonist efficacy in rat injured carotid artery. *J Cardiovasc Pharmacol*, 35, 693-9.
- LERMAN, L. O. & LERMAN, A. (2007) All oxidase roads lead to angiotensin, too. *Arterioscler Thromb Vasc Biol*, 27, 703-4.

- LEUNG, P. S. (2004) The peptide hormone angiotensin II: its new functions in tissues and organs. *Curr Protein Pept Sci*, 5, 267-73.
- LI, J. M. & SHAH, A. M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol*, 287, R1014-30.
- LIU, C., FIGTREE, G. A., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F. & RAMUSSEN, H. H. (2008) Oxidative signalling inhibits the cardiac Na⁺-K⁺ pump via glutathionylation. *submitted*.
- LIU, L. & ASKARI, A. (2006) beta-Subunit of cardiac Na⁺-K⁺-ATPase dictates the concentration of the functional enzyme in caveolae. *Am J Physiol Cell Physiol*, 291, C569-78.
- LIU, L., MOHAMMADI, K., AYNAFSHAR, B., WANG, H., LI, D., LIU, J., IVANOV, A. V., XIE, Z. & ASKARI, A. (2003) Role of caveolae in signal-transducing function of cardiac Na⁺/K⁺-ATPase. *Am J Physiol Cell Physiol*, 284, C1550-60.
- LUPFERT, C., GRELL, E., PINTSCHOVIVUS, V., APELL, H. J., CORNELIUS, F. & CLARKE, R. J. (2001) Rate limitation of the Na⁺,K⁺-ATPase pump cycle. *Biophys J*, 81, 2069-81.
- LUTSENKO, S. & KAPLAN, J. H. (1995) Organization of P-type ATPases: significance of structural diversity. *Biochemistry*, 34, 15607-13.
- MAACK, C., KARTES, T., KILTER, H., SCHAFERS, H. J., NICKENIG, G., BOHM, M. & LAUFS, U. (2003) Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation*, 108, 1567-74.
- MALIK, N., CANFIELD, V. A., BECKERS, M. C., GROS, P. & LEVENSON, R. (1996) Identification of the mammalian Na,K-ATPase 3 subunit. *J Biol Chem*, 271, 22754-8.
- MANN, G. E., YUDILEVICH, D. L. & SOBREVIA, L. (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev*, 83, 183-252.
- MARGOLIS, A. S., PORASUPHATANA, S. & ROSEN, G. M. (2000) Role of paraquat in the uncoupling of nitric oxide synthase. *Biochim Biophys Acta*, 1524, 253-7.
- MARTIN-VASALLO, P., DACKOWSKI, W., EMANUEL, J. R. & LEVENSON, R. (1989) Identification of a putative isoform of the Na,K-ATPase beta subunit. Primary structure and tissue-specific expression. *J Biol Chem*, 264, 4613-8.
- MARTINEZ-RUIZ, A. & LAMAS, S. (2007) Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences. *Cardiovasc Res*, 75, 220-8.
- MASSION PB, F. O., DESSY C, BALLIGAND JL (2003) Nitric oxide and cardiac function: ten years after, and continuing. *Circ Res*. . , 93, 388-98.
- MATHERS, J., FRASER, J. A., MCMAHON, M., SAUNDERS, R. D., HAYES, J. D. & MCLELLAN, L. I. (2004) Antioxidant and cytoprotective responses to redox stress. *Biochem Soc Symp*, 157-76.
- MATSUDA, H. & IYANAGI, T. (1999) Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. *Biochim Biophys Acta*, 1473, 345-55.

- MAYER, B., JOHN, M., HEINZEL, B., WERNER, E. R., WACHTER, H., SCHULTZ, G. & BOHME, E. (1991) Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett*, 288, 187-91.
- MERCER, R. W., BIEMESDERFER, D., BLISS, D. P., JR., COLLINS, J. H. & FORBUSH, B., 3RD (1993) Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol*, 121, 579-86.
- MIHAILIDOU, A. S., BUNDGAARD, H., MARDINI, M., HANSEN, P. S., KJELDEN, K. & RASMUSSEN, H. H. (2000) Hyperaldosteronemia in rabbits inhibits the cardiac sarcolemmal Na(+)-K(+) pump. *Circ Res*, 86, 37-42.
- MOHAN, P., BRUTSAERT, D. L., PAULUS, W. J. & SYS, S. U. (1996) Myocardial contractile response to nitric oxide and cGMP. *Circulation*, 93, 1223-9.
- MORGAN T (2003) Renin, angiotensin, sodium and organ damage. *Hypertens Res*, 26, 349-54.
- MORTH, J. P., PEDERSEN, B. P., TOUSTRUP-JENSEN, M. S., SORENSEN, T. L., PETERSEN, J., ANDERSEN, J. P., VILSEN, B. & NISSEN, P. (2007) Crystal structure of the sodium-potassium pump. *Nature*, 450, 1043-9.
- MUDD, J. O. & KASS, D. A. (2008) Tackling heart failure in the twenty-first century. *Nature*, 451, 919-28.
- MUNSON, K., LAMBRECHT, N., SHIN, J. M. & SACHS, G. (2000) Analysis of the membrane domain of the gastric H(+)/K(+)-ATPase. *J Exp Biol*, 203, 161-70.
- MURDOCH, C. E., GRIEVE, D. J., CAVE, A. C., LOOI, Y. H. & SHAH, A. M. (2006) NADPH oxidase and heart failure. *Curr Opin Pharmacol*, 6, 148-53.
- MURIEL, P., CASTANEDA, G., ORTEGA, M. & NOEL, F. (2003) Insights into the mechanism of erythrocyte Na⁺/K⁺-ATPase inhibition by nitric oxide and peroxy nitrite anion. *J Appl Toxicol*, 23, 275-8.
- MURIEL P, S. G. (2000) Nitric oxide and peroxy nitrite anion modulate liver plasma membrane fluidity and Na⁺/K⁺-ATPase activity. *Nitric Oxide*, 4, 333-342.
- NAKAO, M. & GADSBY, D. C. (1989) [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 539-65.
- NEUBAUER, S. (2007) The failing heart--an engine out of fuel. *N Engl J Med*, 356, 1140-51.
- OHARA, Y., PETERSON, T. E. & HARRISON, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*, 91, 2546-51.
- PACKER, M., CARVER, J. R., RODEHEFFER, R. J., IVANHOE, R. J., DIBIANCO, R., ZELDIS, S. M., HENDRIX, G. H., BOMMER, W. J., ELKAYAM, U., KUKIN, M. L. & ET AL. (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. *N Engl J Med*, 325, 1468-75.

- PARK, J. W., HOYAL, C. R., BENNA, J. E. & BABIOR, B. M. (1997) Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(PHOX) during oxidase activation. *J Biol Chem*, 272, 11035-43.
- PELUFFO, R. D., ARGUELLO, J. M. & BERLIN, J. R. (2000) The role of Na,K-ATPase alpha subunit serine 775 and glutamate 779 in determining the extracellular K⁺ and membrane potential-dependent properties of the Na,K-pump. *J Gen Physiol*, 116, 47-59.
- PELUFFO, R. D., HARA, Y. & BERLIN, J. R. (2004) Quaternary organic amines inhibit Na,K pump current in a voltage-dependent manner: direct evidence of an extracellular access channel in the Na,K-ATPase. *J Gen Physiol*, 123, 249-63.
- PETRUSHANKO, I., BOGDANOV, N., BULYGINA, E., GRENACHER, B., LEINSOO, T., BOLDYREV, A., GASSMANN, M. & BOGDANOVA, A. (2006) Na-K-ATPase in rat cerebellar granule cells is redox sensitive. *Am J Physiol Regul Integr Comp Physiol*, 290, R916-25.
- PIESKE B, MAIER LS, PIACENTINO V 3RD, WEISSER J, HASENFUSS G & HOUSER S (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation.*, 106, 447-453.
- PIESKE, B. & HOUSER, S. R. (2003) [Na⁺]_i handling in the failing human heart. *Cardiovasc Res*, 57, 874-86.
- PIESKE, B., HOUSER, S. R., HASENFUSS, G. & BERS, D. M. (2003) Sodium and the heart: a hidden key factor in cardiac regulation. *Cardiovasc Res*, 57, 871-2.
- PIESKE, B., MAIER, L. S., PIACENTINO, V., 3RD, WEISSER, J., HASENFUSS, G. & HOUSER, S. (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*, 106, 447-53.
- PLUMB, R. D., EL-SHERBEENY, N. A., DIXON, L. J., HUGHES, S. M., DEVINE, A. B., LEAHEY, W. J. & MCVEIGH, G. E. (2005) NAD(P)H-dependent superoxide production in platelets: the role of angiotensin II and protein kinase C. *Clin Biochem*, 38, 607-13.
- POGWIZD SM, SIPIDO KR, VERDONCK F & BERS DM (2003) Intracellular Na in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. *Cardiovasc Res*, 57, 887-896.
- RAJU, S. V., BAROUCH, L. A. & HARE, J. M. (2005) Nitric oxide and oxidative stress in cardiovascular aging. *Sci Aging Knowledge Environ*, 2005, re4.
- RAKOWSKI, R. F., BEZANILLA, F., DE WEER, P., GADSBY, D. C., HOLMGREN, M. & WAGG, J. (1997a) Charge translocation by the Na/K pump. *Ann N Y Acad Sci*, 834, 231-43.
- RAKOWSKI, R. F., GADSBY, D. C. & DE WEER, P. (1997b) Voltage dependence of the Na/K pump. *J Membr Biol*, 155, 105-12.
- RAO, G. N., SARDET, C., POUYSSEGUR, J. & BERK, B. C. (1990) Differential regulation of Na⁺/H⁺ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J Biol Chem*, 265, 19393-6.
- REY, F. E., CIFUENTES, M. E., KIARASH, A., QUINN, M. T. & PAGANO, P. J. (2001) Novel competitive inhibitor of NAD(P)H oxidase assembly

- attenuates vascular O₂(-) and systolic blood pressure in mice. *Circ Res*, 89, 408-14.
- ROMERO-ALVIRA, D. & ROCHE, E. (1996) High blood pressure, oxygen radicals and antioxidants: etiological relationships. *Med Hypotheses*, 46, 414-20.
- RUSSELL, J. M. (2000) Sodium-potassium-chloride cotransport. *Physiol Rev*, 80, 211-76.
- RYBIN, V. O., XU, X. & STEINBERG, S. F. (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae : stimulation of local protein phosphorylation. *Circ Res*, 84, 980-8.
- SAGAR, A. & RAKOWSKI, R. F. (1994) Access channel model for the voltage dependence of the forward-running Na⁺/K⁺ pump. *J Gen Physiol*, 103, 869-93.
- SARCEVIC, B., BROOKES, V., MARTIN, T. J., KEMP, B. E. & ROBINSON, P. J. (1989) Atrial natriuretic peptide-dependent phosphorylation of smooth muscle cell particulate fraction proteins is mediated by cGMP-dependent protein kinase. *J Biol Chem*, 264, 20648-54.
- SATO, A., YAGIHARA, N., KODAMA, M., MITSUMA, W., TACHIKAWA, H., ITO, M., HANAWA, H. & AIZAWA, Y. Takotsubo cardiomyopathy after delivery in an oestrogen-deficient patient. *Int J Cardiol*, 149, e78-9.
- SATO, T., KAMATA, Y., IRIFUNE, M. & NISHIKAWA, T. (1997) Inhibitory effect of several nitric oxide-generating compounds on purified Na⁺,K⁽⁺⁾-ATPase activity from porcine cerebral cortex. *J Neurochem*, 68, 1312-8.
- SEDDON, M., LOOI, Y. H. & SHAH, A. M. (2007) Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart*, 93, 903-7.
- SHAH, A. M. & MACCARTHY, P. A. (2000) Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther*, 86, 49-86.
- SHAINSKAYA, A., SCHNEEBERGER, A., APELL, H. J. & KARLISH, S. J. (2000) Entrance port for Na⁽⁺⁾ and K⁽⁺⁾ ions on Na⁽⁺⁾,K⁽⁺⁾-ATPase in the cytoplasmic loop between trans-membrane segments M6 and M7 of the alpha subunit. Proximity Of the cytoplasmic segment of the beta subunit. *J Biol Chem*, 275, 2019-28.
- SHAMRAJ, O. I. & LINGREL, J. B. (1994) A putative fourth Na⁺,K⁽⁺⁾-ATPase alpha-subunit gene is expressed in testis. *Proc Natl Acad Sci U S A*, 91, 12952-6.
- SHIMON, M. B., GOLDSHLEGER, R. & KARLISH, S. J. (1998) Specific Cu²⁺-catalyzed oxidative cleavage of Na,K-ATPase at the extracellular surface. *J Biol Chem*, 273, 34190-5.
- SHINODA, T., OGAWA, H., CORNELIUS, F. & TOYOSHIMA, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature*, 459, 446-50.
- SILVERMAN, B. Z., FULLER, W., EATON, P., DENG, J., MOORMAN, J. R., CHEUNG, J. Y., JAMES, A. F. & SHATTOCK, M. J. (2005) Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. *Cardiovasc Res*, 65, 93-103.
- SIRAGY, H. M. & CAREY, R. M. (1997) The subtype 2 (AT₂) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*, 100, 264-9.

- SKOU, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta*, 23, 394-401.
- SKOU, J. C. (1998) Nobel Lecture. The identification of the sodium pump. *Biosci Rep*, 18, 155-69.
- SOMERS, M. J., MAVROMATIS, K., GALIS, Z. S. & HARRISON, D. G. (2000) Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation*, 101, 1722-8.
- STADTMAN, E. R. & LEVINE, R. L. (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, 25, 207-18.
- STANTON, B. A. & KAISLING, B. (1989) Regulation of renal ion transport and cell growth by sodium. *Am J Physiol*, 257, F1-10.
- STOLK, J., HILTERMANN, T. J., DIJKMAN, J. H. & VERHOEVEN, A. J. (1994) Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*, 11, 95-102.
- STUEHR, D. J., CHO, H. J., KWON, N. S., WEISE, M. F. & NATHAN, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, 88, 7773-7.
- SWEADNER, K. J. (2005) Phospholemman: a new force in cardiac contractility. *Circ Res*, 97, 510-1.
- SWEADNER, K. J., ARYSTARKHOVA, E., DONNET, C. & WETZEL, R. K. (2003) FXYD proteins as regulators of the Na,K-ATPase in the kidney. *Ann N Y Acad Sci*, 986, 382-7.
- SWEADNER, K. J. & FESCHENKO, M. S. (2001) Predicted location and limited accessibility of protein kinase A phosphorylation site on Na-K-ATPase. *Am J Physiol Cell Physiol*, 280, C1017-26.
- SWEADNER, K. J. & RAEL, E. (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics*, 68, 41-56.
- SWEDBERG, K., ENEROTH, P., KJEKSHUS, J. & WILHELMSEN, L. (1990) Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group. *Circulation*, 82, 1730-6.
- SYDOW, K. & MUNZEL, T. (2003) ADMA and oxidative stress. *Atheroscler Suppl*, 4, 41-51.
- SZABO, C. (2003) Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett*, 140-141, 105-12.
- TAMURA, N., KONISHI, S., IWAKI, S., KIMURA-SOMEYA, T., NADA, S. & YAMAGUCHI, A. (2001) Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J Biol Chem*, 276, 20330-9.
- TAUB, M., BORSICK, M., GEISEL, J., MATLHAGELA, K., RAJKHOWA, T. & ALLEN, C. (2004) Regulation of the Na,K-ATPase in MDCK cells by prostaglandin E1: a role for calcium as well as cAMP. *Exp Cell Res*, 299, 1-14.

- TEN HOVE, M., CHAN, S., LYGATE, C., MONFARED, M., BOEHM, E., HULBERT, K., WATKINS, H., CLARKE, K. & NEUBAUER, S. (2005) Mechanisms of creatine depletion in chronically failing rat heart. *J Mol Cell Cardiol*, 38, 309-13.
- THERIEN, A. G. & BLOSTEIN, R. (1999) K(+)/Na(+) antagonism at cytoplasmic sites of Na(+)-K(+)-ATPase: a tissue-specific mechanism of sodium pump regulation. *Am J Physiol*, 277, C891-8.
- THERIEN, A. G. & BLOSTEIN, R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*, 279, C541-66.
- THERIEN AG, B. R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*, 279, 541-66.
- TODA, N., AYAJIKI, K. & OKAMURA, T. (2007) Interaction of endothelial nitric oxide and angiotensin in the circulation. *Pharmacol Rev*, 59, 54-87.
- TOURNEUR, Y., MITRA, R., MORAD, M. & ROUGIER, O. (1987) Activation properties of the inward-rectifying potassium channel on mammalian heart cells. *J Membr Biol*, 97, 127-35.
- TSUTSUI, H. (2004) Novel Pathophysiological Insight and Treatment Strategies for Heart Failure. *Circ J*, 68, 1095-1103.
- TURKO, I. V. & MURAD, F. (2002) Protein nitration in cardiovascular diseases. *Pharmacol Rev*, 54, 619-34.
- VARELA, M., HERRERA, M. & GARVIN, J. L. (2004) Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on O₂- and is diminished by a high-salt diet. *Am J Physiol Renal Physiol*, 287, F224-30.
- VASQUEZ-VIVAR, J., MARTASEK, P., WHITSETT, J., JOSEPH, J. & KALYANARAMAN, B. (2002) The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J*, 362, 733-9.
- VAUGHAN-JONES, R. D., EISNER, D. A. & LEDERER, W. J. (1985) The effects of intracellular Na on contraction and intracellular pH in mammalian cardiac muscle. *Adv Myocardiol*, 5, 313-30.
- VIGNERY, A., WANG, F., QIAN, H. Y., BENZ, E. J., JR. & GILMORE-HEBERT, M. (1991) Detection of the Na(+)-K(+)-ATPase alpha 3-isoform in multinucleated macrophages. *Am J Physiol*, 260, F704-9.
- VILA-PETROFF, M. G., YOUNES, A., EGAN, J., LAKATTA, E. G. & SOLLOTT, S. J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res*, 84, 1020-31.
- VUKOSAVLJEVIC, N., JARON, D., BARBEE, K. A. & BUERK, D. G. (2006) Quantifying the L-arginine paradox in vivo. *Microvasc Res*, 71, 48-54.
- WANG, F., CHEN, C. C., MAO, B. Y., MAO, Q., ZHANG, Y. K., LEI, D. & XU, R. X. (2004) [Effect of L-arginine on the changes of cerebral microcirculation following craniocerebral missile wound at early stage in cats]. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 35, 402-5.
- WANG, Y., GAO, J., MATHIAS, R. T., COHEN, I. S., SUN, X. & BALDO, G. J. (1998) alpha-Adrenergic effects on Na⁺-K⁺ pump current in guinea-pig ventricular myocytes. *J Physiol*, 509 (Pt 1), 117-28.
- WARNHOLTZ, A., NICKENIG, G., SCHULZ, E., MACHARZINA, R., BRASEN, J. H., SKATCHKOV, M., HEITZER, T., STASCH, J. P., GRIENDLING, K. K., HARRISON, D. G., BOHM, M., MEINERTZ, T. & MUNZEL, T. (1999)

- Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*, 99, 2027-33.
- WENZEL-SEIFERT, K., LIU, H. Y. & SEIFERT, R. (2002) Similarities and differences in the coupling of human beta1- and beta2-adrenoceptors to Gs(alpha) splice variants. *Biochem Pharmacol*, 64, 9-20.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., HAMILTON, E. J., GARCIA, A., WANG, D., CHIA, K. K. M., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) Opposing effects of coupled and uncoupled NOS activity on the Na⁺-K⁺ pump in cardiac myocytes. *Am J Physiol Cell Physiol*, 294, C572-8.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2010) Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008a) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation. *Am J Physiol Cell Physiol*, 294, C1067-73.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*, 565, 815-25.
- WILLIAMS, H. C. & GRIENGLING, K. K. (2007) NADPH oxidase inhibitors: new antihypertensive agents? *J Cardiovasc Pharmacol*, 50, 9-16.
- WINTERBOURN, C. C. & HAMPTON, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med*, 45, 549-61.
- XIE Z, KOMETIANI P, LIU J, LI J, SHAPIRO JI & ASKARI A (1999) Intracellular reactive oxygen species mediate the linkage of Na⁺/K⁺-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem.*, 274, 19323-19328.
- YANG, B. & RIZZO, V. (2007) TNF- α potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*, 292, H954-962.
- YAO, L., FAN, P., JIANG, Z., GORDON, A., MOCHLY-ROSEN, D. & DIAMOND, I. (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol*, 73, 1105-12.

- ZAUGG, M., LUCCHINETTI, E., UECKER, M., PASCH, T. & SCHAUB, M. C. (2003) Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth*, 91, 551-65.
- ZHANG, C., IMAM, S. Z., ALI, S. F. & MAYEUX, P. R. (2002) Peroxynitrite and the regulation of Na(+),K(+)-ATPase activity by angiotensin II in the rat proximal tubule. *Nitric Oxide*, 7, 30-5.
- ZHAO, X., XU, B., BHATTACHARJEE, A., OLDFIELD, C. M., WIENTJES, F. B., FELDMAN, G. M. & CATHCART, M. K. (2005) Protein kinase Cdelta regulates p67phox phosphorylation in human monocytes. *J Leukoc Biol*, 77, 414-20.
- (1990) Xamoterol in severe heart failure. The Xamoterol in Severe Heart Failure Study Group. *Lancet*, 336, 1-6.
- ABEL, E. D. (2004) Glucose transport in the heart. *Front Biosci*, 9, 201-15.
- ADACHI, T., WEISBROD, R. M., PIMENTEL, D. R., YING, J., SHAROV, V. S., SCHONEICH, C. & COHEN, R. A. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med*, 10, 1200-7.
- ALDERTON WK, COOPER CE & KNOWLES RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J.*, 357, 593-615.
- ANVERSA, P., LERI, A., BELTRAMI, C. A., GUERRA, S. & KAJSTURA, J. (1998) Myocyte death and growth in the failing heart. *Lab Invest*, 78, 767-86.
- APELL, H. J. & KARLISH, S. J. (2001) Functional properties of Na,K-ATPase, and their structural implications, as detected with biophysical techniques. *J Membr Biol*, 180, 1-9.
- BABIOR, B. M. (2000) The NADPH oxidase of endothelial cells. *IUBMB Life*, 50, 267-9.
- BAHINSKI, A., NAIRN, A. C., GREENGARD, P. & GADSBY, D. C. (1989) Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*, 340, 718-21.
- BALLIGAND, J. L. (2000) [Beta 3 adrenergic receptor: physiologic role and potential therapeutic applications]. *Bull Mem Acad R Med Belg*, 155, 311-7; discussion 317-9.
- BALLIGAND, J. L., UNGUREANU-LONGROIS, D., SIMMONS, W. W., PIMENTAL, D., MALINSKI, T. A., KAPTURCZAK, M., TAHA, Z., LOWENSTEIN, C. J., DAVIDOFF, A. J., KELLY, R. A. & ET AL. (1994) Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem*, 269, 27580-8.
- BAROUCH, L. A., HARRISON, R. W., SKAF, M. W., ROSAS, G. O., CAPPOLA, T. P., KOBEISSI, Z. A., HOBBI, I. A., LEMMON, C. A., BURNETT, A. L., O'ROURKE, B., RODRIGUEZ, E. R., HUANG, P. L., LIMA, J. A., BERKOWITZ, D. E. & HARE, J. M. (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature*, 416, 337-9.
- BARWE, S. P., KIM, S., RAJASEKARAN, S. A., BOWIE, J. U. & RAJASEKARAN, A. K. (2007) Janus model of the Na,K-ATPase beta-

- subunit transmembrane domain: distinct faces mediate alpha/beta assembly and beta-beta homo-oligomerization. *J Mol Biol*, 365, 706-14.
- BEDARD, K. & KRAUSE, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*, 87, 245-313.
- BERK, B. C., ELDER, E. & MITSUKA, M. (1990) Hypertrophy and hyperplasia cause differing effects on vascular smooth muscle cell Na⁺/H⁺ exchange and intracellular pH. *J Biol Chem*, 265, 19632-7.
- BERS, D. M. (2001) *Excitation-Contraction Coupling and Cardiac Contractile Force* Dordrecht, The Netherlands, Kluwer Academic Press:427.
- BERS, D. M., BARRY, W. H. & DESPA, S. (2003) Intracellular Na⁺ regulation in cardiac myocytes. *Cardiovasc Res*, 57, 897-912.
- BIBERT, S., ROY, S., SCHAER, D., HORISBERGER, J. D. & GEERING, K. (2008) Phosphorylation of phospholemman (FXD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *J Biol Chem*, 283, 476-86.
- BLANCO, G., SANCHEZ, G., MELTON, R. J., TOURTELLOTTE, W. G. & MERCER, R. W. (2000) The alpha4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *J Histochem Cytochem*, 48, 1023-32.
- BOLDYREV, A. A., BULYGINA, E. R., KRAMARENKO, G. G. & VANIN, A. F. (1997) Effect of nitroso compounds on Na/K-ATPase. *Biochim Biophys Acta*, 1321, 243-51.
- BORGES, V. C., ROCHA, J. B. & NOGUEIRA, C. W. (2005) Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. *Toxicology*, 215, 191-7.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001a) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na(+)-K(+) pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001b) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na⁺-K⁺ pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., GRAY, D. F., MIHAILIDOU, A. S. & RASMUSSEN, H. H. (1999) Angiotensin regulates the selectivity of the Na⁺-K⁺ pump for intracellular Na⁺. *Am J Physiol*, 277, C461-8.
- BUHAGIAR, K. A., HANSEN, P. S., KONG, B. Y., CLARKE, R. J., FERNANDES, C. & RASMUSSEN, H. H. (2004) Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes. *Am J Physiol Cell Physiol*, 286, C398-405.
- BUNDGAARD H, G. A., HAMILTON EJ, WHITE CN, RASMUSSEN HH (2006) Beta-3 receptors mediate adrenergic stimulation of the sarcolemmal Na⁺-K⁺ pump. *Biophys J suppl*, 556a.
- BUTLER, R., MORRIS, A. D., BELCH, J. J., HILL, A. & STRUTHERS, A. D. (2000) Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension*, 35, 746-51.
- CAI, H., GRIENDLING, K. K. & HARRISON, D. G. (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*, 24, 471-8.

- CAI, H. & HARRISON, D. G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*, 87, 840-4.
- CAMBIER, J. C., NEWELL, M. K., JUSTEMENT, L. B., MCGUIRE, J. C., LEACH, K. L. & CHEN, Z. Z. (1987) Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature*, 327, 629-32.
- CAPPOLA, T. P., KASS, D. A., NELSON, G. S., BERGER, R. D., ROSAS, G. O., KOBEISSI, Z. A., MARBAN, E. & HARE, J. M. (2001) Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation*, 104, 2407-11.
- CHARPENTIER, F., LEGATO, M. J., STEINBERG, S. F., COHEN, I. S. & ROSEN, M. R. (1996) Beta-adrenergic modulation of Na-K pump activity in young and adult canine cardiac Purkinje fibers. *Am J Physiol*, 271, H706-12.
- CHENG, X. J., HOOG, J. O., NAIRN, A. C., GREENGARD, P. & APERIA, A. (1997) Regulation of rat Na(+)-K(+)-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am J Physiol*, 273, C1981-6.
- CLAUSEN, T., VAN HARDEVELD, C. & EVERTS, M. E. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev*, 71, 733-74.
- CLAVREUL, N., BACHSCHMID, M. M., HOU, X., SHI, C., IDRIZOVIC, A., IDO, Y., PIMENTEL, D. & COHEN, R. A. (2006) S-glutathiolation of p21ras by peroxynitrite mediates endothelial insulin resistance caused by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol*, 26, 2454-61.
- CLOSS, E. I., SCHELD, J. S., SHARAFI, M. & FORSTERMANN, U. (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol*, 57, 68-74.
- COHEN, R. A. & ADACHI, T. (2006) Nitric-oxide-induced vasodilatation: regulation by physiologic s-glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc Med*, 16, 109-14.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003a) Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci*, 18, 119-24.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003b) Themes in ion pump regulation. *Ann N Y Acad Sci*, 986, 579-86.
- CORNELIUS, F., MAHMMOUD, Y. A. & CHRISTENSEN, H. R. (2001) Modulation of Na,K-ATPase by associated small transmembrane regulatory proteins and by lipids. *J Bioenerg Biomembr*, 33, 415-23.
- DAIBER, A., ZOU, M. H., BACHSCHMID, M. & ULLRICH, V. (2000) Ebselen as a peroxynitrite scavenger in vitro and ex vivo. *Biochem Pharmacol*, 59, 153-60.
- DE GASPARO, M. & SIRAGY, H. M. (1999) The AT2 receptor: fact, fancy and fantasy. *Regul Pept*, 81, 11-24.
- DESPA, S., BOSSUYT, J., HAN, F., GINSBURG, K. S., JIA, L. G., KUTCHAI, H., TUCKER, A. L. & BERS, D. M. (2005) Phospholemmam-

- phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. *Circ Res*, 97, 252-9.
- DESPA, S., ISLAM, M. A., WEBER, C. R., POGWIZD, S. M. & BERS, D. M. (2002) Intracellular Na(+) concentration is elevated in heart failure but Na/K pump function is unchanged. *Circulation*, 105, 2543-8.
- DOEGE, H., BOCIANSKI, A., SCHEEPERS, A., AXER, H., ECKEL, J., JOOST, H. G. & SCHURMANN, A. (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J*, 359, 443-9.
- DUDZINSKI, D. M., IGARASHI, J., GREIF, D. & MICHEL, T. (2006) The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol*, 46, 235-76.
- EKELUND, U. E., HARRISON, R. W., SHOKEK, O., THAKKAR, R. N., TUNIN, R. S., SENZAKI, H., KASS, D. A., MARBAN, E. & HARE, J. M. (1999) Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circ Res*, 85, 437-45.
- ELLIS, D. Z., RABE, J. & SWEADNER, K. J. (2003) Global loss of Na,K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci*, 23, 43-51.
- ERLENKAMP, S., GLITSCH, H. G. & KOCKSKAMPER, J. (2002) Dual regulation of cardiac Na⁺-K⁺ pumps and CFTR Cl⁻ channels by protein kinases A and C. *Pflugers Arch*, 444, 251-62.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009a) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009b) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FLIEGEL, L. (2008) Molecular biology of the myocardial Na⁺/H⁺ exchanger. *J Mol Cell Cardiol*, 44, 228-37.
- FLIEGEL, L. & FROHLICH, O. (1993) The Na⁺/H⁺ exchanger: an update on structure, regulation and cardiac physiology. *Biochem J*, 296 (Pt 2), 273-85.
- FOLLMER, C. H., TEN EICK, R. E. & YEH, J. Z. (1987) Sodium current kinetics in cat atrial myocytes. *J Physiol*, 384, 169-97.
- FORBUSH, B., 3RD, KAPLAN, J. H. & HOFFMAN, J. F. (1978) Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry*, 17, 3667-76.
- FRIEBE, A. & KOESLING, D. (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res*, 93, 96-105.
- FULLER, W., EATON, P., BELL, J. R. & SHATTOCK, M. J. (2004) Ischemia-induced phosphorylation of phospholemman directly activates rat cardiac Na/K-ATPase. *Faseb J*, 18, 197-9.

- FULLER, W., HOWIE, J., MCLATCHIE, L. M., WEBER, R. J., HASTIE, C. J., BURNES, K., PAVLOVIC, D. & SHATTOCK, M. J. (2009) FXD1 phosphorylation in vitro and in adult rat cardiac myocytes: threonine 69 is a novel substrate for protein kinase C. *Am J Physiol Cell Physiol*, 296, C1346-55.
- GADSBY, D. C. (2007) Structural biology: ion pumps made crystal clear. *Nature*, 450, 957-9.
- GADSBY, D. C., KIMURA, J. & NOMA, A. (1985) Voltage dependence of Na/K pump current in isolated heart cells. *Nature*, 315, 63-5.
- GADSBY, D. C. & NAKAO, M. (1989) Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 511-37.
- GADSBY, D. C., NAKAO, M. & BAHINSKI, A. (1989) Voltage dependence of transient and steady-state Na/K pump currents in myocytes. *Mol Cell Biochem*, 89, 141-6.
- GALLOGLY, M. M. & MIEYAL, J. J. (2007) Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr Opin Pharmacol*, 7, 381-91.
- GAO, J., COHEN, I. S., MATHIAS, R. T. & BALDO, G. J. (1998) The inhibitory effect of beta-stimulation on the Na/K pump current in guinea pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. *Pflugers Arch*, 435, 479-84.
- GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1992) Isoprenaline, Ca²⁺ and the Na(+)-K+ pump in guinea-pig ventricular myocytes. *J Physiol*, 449, 689-704.
- GAO, J., MATHIAS, R. T., COHEN, I. S., WANG, Y., SUN, X. & BALDO, G. J. (1999) Activation of PKC increases Na⁺-K⁺ pump current in ventricular myocytes from guinea pig heart. *Pflugers Arch*, 437, 643-51.
- GARCIA, A., BUNDGAARD, H., HAMILTON, E., LIU, C., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) beta-3 adrenergic stimulation of the Na⁺-K⁺ pump is mediated by NO-dependent de-glutathionylation of the beta-1 pump subunit. *12th International ATPase Conference*. Aarhus.
- GARTY, H. & KARLISH, S. J. (2006) Role of FXD proteins in ion transport. *Annu Rev Physiol*, 68, 431-59.
- GEERING, K. (2001) The functional role of beta subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr*, 33, 425-38.
- GHEZZI, P. (2005) Regulation of protein function by glutathionylation. *Free Radic Res*, 39, 573-80.
- GLITSCH, H. G. (2001) Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev*, 81, 1791-826.
- GOLIGORSKY, M. S., CHEN, J. & BRODSKY, S. (2001) Workshop: endothelial cell dysfunction leading to diabetic nephropathy : focus on nitric oxide. *Hypertension*, 37, 744-8.
- GOLIGORSKY, M. S. & GROSS, S. S. (2001) The ins and outs of endothelial dysfunction: much ado about NO-thing. *Drug News Perspect*, 14, 133-42.
- GRIENGLING, K. K., MINIERI, C. A., OLLERENSHAW, J. D. & ALEXANDER, R. W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*, 74, 1141-8.

- GRIENGLING, K. K., SORESCU, D. & USHIO-FUKAI, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*, 86, 494-501.
- GUIMBAL, C. & KILIMANN, M. W. (1993) A Na(+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem*, 268, 8418-21.
- GUTHIKONDA, S., SINKEY, C., BARENZ, T. & HAYNES, W. G. (2003) Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers. *Circulation*, 107, 416-21.
- HAN, F., BOSSUYT, J., DESPA, S., TUCKER, A. L. & BERS, D. M. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na⁺/K⁺ pump function in cardiac myocytes. *Circ Res*, 99, 1376-83.
- HAN, J., KIM, N., KIM, E., HO, W. K. & EARM, Y. E. (2001) Modulation of ATP-sensitive potassium channels by cGMP-dependent protein kinase in rabbit ventricular myocytes. *J Biol Chem*, 276, 22140-7.
- HANSEN, P. S., BUHAGIAR, K. A., KONG, B. Y., CLARKE, R. J., GRAY, D. F. & RASMUSSEN, H. H. (2002) Dependence of Na⁺-K⁺ pump current-voltage relationship on intracellular Na⁺, K⁺, and Cs⁺ in rabbit cardiac myocytes. *Am J Physiol Cell Physiol*, 283, C1511-21.
- HANSEN, P. S., CLARKE, R. J., BUHAGIAR, K. A., HAMILTON, E., GARCIA, A., WHITE, C. & RASMUSSEN, H. H. (2007) Alloxan-induced diabetes reduces sarcolemmal Na⁺-K⁺ pump function in rabbit ventricular myocytes. *Am J Physiol Cell Physiol*, 292, C1070-1077.
- HARADA, K., LIN, H., ENDO, Y., FUJISHIRO, N., SAKAMOTO, Y. & INOUE, M. (2006) Subunit composition and role of Na⁺,K⁺-ATPases in ventricular myocytes. *J Physiol Sci*, 56, 113-21.
- HARRISON, D. G. (1997) Endothelial function and oxidant stress. *Clin Cardiol*, 20, II-11-7.
- HASLER, U., CRAMBERT, G., HORISBERGER, J. D. & GEERING, K. (2001) Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. *J Biol Chem*, 276, 16356-64.
- HELIN, P. & LORENZEN, I. (1976) Seasonal variations in the susceptibility of the aortic wall to atherosclerosis. Biochemical studies of glycosaminoglycans and collagen of rabbit atherosclerosis. *Atherosclerosis*, 24, 259-66.
- HERRERA, V. L., EMANUEL, J. R., RUIZ-OPAZO, N., LEVENSON, R. & NADAL-GINARD, B. (1987) Three differentially expressed Na,K-ATPase alpha subunit isoforms: structural and functional implications. *J Cell Biol*, 105, 1855-65.
- HESS, D. T., MATSUMOTO, A., KIM, S. O., MARSHALL, H. E. & STAMLER, J. S. (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*, 6, 150-66.
- HEVEL, J. M. & MARLETTA, M. A. (1992) Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry*, 31, 7160-5.
- HINGTGEN, S. D., TIAN, X., YANG, J., DUNLAY, S. M., PEEK, A. S., WU, Y., SHARMA, R. V., ENGELHARDT, J. F. & DAVISSON, R. L. (2006) Nox2-

- containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. *Physiol Genomics*, 26, 180-91.
- HOFFMANN, C., LEITZ, M. R., OBERDORF-MAASS, S., LOHSE, M. J. & KLOTZ, K. N. (2004) Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*, 369, 151-9.
- HOOL LC, GRAY DF, ROBINSON BG & RASMUSSEN HH (1996) Angiotensin-converting enzyme inhibitors regulate the Na(+)-K+ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-C180.
- HOOL, L. C., GRAY, D. F., ROBINSON, B. G. & RASMUSSEN, H. H. (1996) Angiotensin-converting enzyme inhibitors regulate the Na⁺-K⁺ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-80.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995a) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na(+)-K+ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995b) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na⁺-K⁺ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOUSER, S. R. & LAKATTA, E. G. (1999) Function of the cardiac myocyte in the conundrum of end-stage, dilated human heart failure. *Circulation*, 99, 600-4.
- HUANG, B., BLANCO, G., MERCER, R. W., FLEMING, T. & PEPOSE, J. S. (2003) Human corneal endothelial cell expression of Na⁺,K⁺-adenosine triphosphatase isoforms. *Arch Ophthalmol*, 121, 840-5.
- HUANG, N. K., LIN, Y. W., HUANG, C. L., MESSING, R. O. & CHERN, Y. (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. *J Biol Chem*, 276, 13838-46.
- IDE, T., TSUTSUI, H., KINUGAWA, S., UTSUMI, H., KANG, D., HATTORI, N., UCHIDA, K., ARIMURA, K., EGASHIRA, K. & TAKESHITA, A. (1999) Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res*, 85, 357-63.
- IKEHARA, T., YAMAGUCHI, H., HOSOKAWA, K., TAKAHASHI, A. & MIYAMOTO, H. (1993) Kinetic study on the effects of intracellular K⁺ and Na⁺ on Na⁺, K⁺, Cl⁻ cotransport of HeLa cells by Rb⁺ influx determination. *J Membr Biol*, 132, 115-24.
- IVANOV, A., ZHAO, H. & MODYANOV, N. N. (2000) Packing of the transmembrane helices of Na,K-ATPase: direct contact between beta-subunit and H8 segment of alpha-subunit revealed by oxidative cross-linking. *Biochemistry*, 39, 9778-85.
- JIA, L. G., DONNET, C., BOGAEV, R. C., BLATT, R. J., MCKINNEY, C. E., DAY, K. H., BERR, S. S., JONES, L. R., MOORMAN, J. R., SWEADNER, K. J. & TUCKER, A. L. (2005) Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice. *Am J Physiol Heart Circ Physiol*, 288, H1982-8.
- JOHNSON, F. & GIULIVI, C. (2005) Superoxide dismutases and their impact upon human health. *Mol Aspects Med*, 26, 340-52.

- KANAI, A. J., MESAROS, S., FINKEL, M. S., ODDIS, C. V., BIRDER, L. A. & MALINSKI, T. (1997) Beta-adrenergic regulation of constitutive nitric oxide synthase in cardiac myocytes. *Am J Physiol*, 273, C1371-7.
- KARLISH, S. J., YATES, D. W. & GLYNN, I. M. (1978) Elementary steps of the (Na⁺ + K⁺)-ATPase mechanism, studied with formycin nucleotides. *Biochim Biophys Acta*, 525, 230-51.
- KLEIN, L., O'CONNOR, C. M., GATTIS, W. A., ZAMPINO, M., DE LUCA, L., VITARELLI, A., FEDELE, F. & GHEORGHIADE, M. (2003) Pharmacologic therapy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations. *Am J Cardiol*, 91, 18F-40F.
- KOCKSKAMPER, J., ERLenkAMP, S. & GLITSCH, H. G. (2000) Activation of the cAMP-protein kinase A pathway facilitates Na⁺ translocation by the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 523 Pt 3, 561-74.
- KOCKSKAMPER, J., SENDHOFF, K., ERLenkAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOCKSKÄMPER, J., SENDHOFF, K., ERLenkAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOJIMA, H., NAKATSUBO, N., KIKUCHI, K., KAWAHARA, S., KIRINO, Y., NAGOSHI, H., HIRATA, Y. & NAGANO, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem*, 70, 2446-53.
- KONG, B. Y. & CLARKE, R. J. (2004) Identification of potential regulatory sites of the Na⁺,K⁺-ATPase by kinetic analysis. *Biochemistry*, 43, 2241-50.
- KOU, R. & MICHEL, T. (2007) Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*, 282, 32719-29.
- KURZ, S. & HARRISON, D. G. (1997) Insulin and the arginine paradox. *J Clin Invest*, 99, 369-70.
- LAMBETH, J. D., KAWAHARA, T. & DIEBOLD, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med*, 43, 319-31.
- LASS, A., SUESSENBACHER, A., WOLKART, G., MAYER, B. & BRUNNER, F. (2002) Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol*, 61, 1081-8.
- LASTRA, G. & MANRIQUE, C. (2007) The expanding role of oxidative stress, renin angiotensin system, and beta-cell dysfunction in the cardiometabolic syndrome and Type 2 diabetes mellitus. *Antioxid Redox Signal*, 9, 943-54.
- LAUGER, P. (1991) Kinetic basis of voltage dependence of the Na,K-pump. *Soc Gen Physiol Ser*, 46, 303-15.
- LEMAY, J., HOU, Y. & DEBLOIS, D. (2000) Evidence that nitric oxide regulates AT1-receptor agonist and antagonist efficacy in rat injured carotid artery. *J Cardiovasc Pharmacol*, 35, 693-9.

- LERMAN, L. O. & LERMAN, A. (2007) All oxidase roads lead to angiotensin, too. *Arterioscler Thromb Vasc Biol*, 27, 703-4.
- LEUNG, P. S. (2004) The peptide hormone angiotensin II: its new functions in tissues and organs. *Curr Protein Pept Sci*, 5, 267-73.
- LI, J. M. & SHAH, A. M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol*, 287, R1014-30.
- LIU, C., FIGTREE, G. A., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F. & RAMUSSEN, H. H. (2008) Oxidative signalling inhibits the cardiac Na⁺-K⁺ pump via glutathionylation. *submitted*.
- LIU, L. & ASKARI, A. (2006) beta-Subunit of cardiac Na⁺-K⁺-ATPase dictates the concentration of the functional enzyme in caveolae. *Am J Physiol Cell Physiol*, 291, C569-78.
- LIU, L., MOHAMMADI, K., AYNAFSHAR, B., WANG, H., LI, D., LIU, J., IVANOV, A. V., XIE, Z. & ASKARI, A. (2003) Role of caveolae in signal-transducing function of cardiac Na⁺/K⁺-ATPase. *Am J Physiol Cell Physiol*, 284, C1550-60.
- LUPFERT, C., GRELL, E., PINTSCHOVIVUS, V., APELL, H. J., CORNELIUS, F. & CLARKE, R. J. (2001) Rate limitation of the Na⁺,K⁺-ATPase pump cycle. *Biophys J*, 81, 2069-81.
- LUTSENKO, S. & KAPLAN, J. H. (1995) Organization of P-type ATPases: significance of structural diversity. *Biochemistry*, 34, 15607-13.
- MAACK, C., KARTES, T., KILTER, H., SCHAFERS, H. J., NICKENIG, G., BOHM, M. & LAUFS, U. (2003) Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation*, 108, 1567-74.
- MALIK, N., CANFIELD, V. A., BECKERS, M. C., GROS, P. & LEVENSON, R. (1996) Identification of the mammalian Na,K-ATPase 3 subunit. *J Biol Chem*, 271, 22754-8.
- MANN, G. E., YUDILEVICH, D. L. & SOBREVIA, L. (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev*, 83, 183-252.
- MARGOLIS, A. S., PORASUPHATANA, S. & ROSEN, G. M. (2000) Role of paraquat in the uncoupling of nitric oxide synthase. *Biochim Biophys Acta*, 1524, 253-7.
- MARTIN-VASALLO, P., DACKOWSKI, W., EMANUEL, J. R. & LEVENSON, R. (1989) Identification of a putative isoform of the Na,K-ATPase beta subunit. Primary structure and tissue-specific expression. *J Biol Chem*, 264, 4613-8.
- MARTINEZ-RUIZ, A. & LAMAS, S. (2007) Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences. *Cardiovasc Res*, 75, 220-8.
- MASSION PB, F. O., DESSY C, BALLIGAND JL (2003) Nitric oxide and cardiac function: ten years after, and continuing. *Circ Res*. , 93, 388-98.
- MATHERS, J., FRASER, J. A., MCMAHON, M., SAUNDERS, R. D., HAYES, J. D. & MCLELLAN, L. I. (2004) Antioxidant and cytoprotective responses to redox stress. *Biochem Soc Symp*, 157-76.

- MATSUDA, H. & IYANAGI, T. (1999) Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. *Biochim Biophys Acta*, 1473, 345-55.
- MAYER, B., JOHN, M., HEINZEL, B., WERNER, E. R., WACHTER, H., SCHULTZ, G. & BOHME, E. (1991) Brain nitric oxide synthase is a bipterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett*, 288, 187-91.
- MERCER, R. W., BIEMESDERFER, D., BLISS, D. P., JR., COLLINS, J. H. & FORBUSH, B., 3RD (1993) Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol*, 121, 579-86.
- MIHAILIDOU, A. S., BUNDGAARD, H., MARDINI, M., HANSEN, P. S., KJELDEN, K. & RASMUSSEN, H. H. (2000) Hyperaldosteronemia in rabbits inhibits the cardiac sarcolemmal Na(+)-K(+) pump. *Circ Res*, 86, 37-42.
- MOHAN, P., BRUTSAERT, D. L., PAULUS, W. J. & SYS, S. U. (1996) Myocardial contractile response to nitric oxide and cGMP. *Circulation*, 93, 1223-9.
- MORGAN T (2003) **Renin, angiotensin, sodium and organ damage.** *Hypertens Res*, 26, 349-54.
- MORTH, J. P., PEDERSEN, B. P., TOUSTRUP-JENSEN, M. S., SORENSEN, T. L., PETERSEN, J., ANDERSEN, J. P., VILSEN, B. & NISSEN, P. (2007) Crystal structure of the sodium-potassium pump. *Nature*, 450, 1043-9.
- MUDD, J. O. & KASS, D. A. (2008) Tackling heart failure in the twenty-first century. *Nature*, 451, 919-28.
- MUNSON, K., LAMBRECHT, N., SHIN, J. M. & SACHS, G. (2000) Analysis of the membrane domain of the gastric H(+)/K(+)-ATPase. *J Exp Biol*, 203, 161-70.
- MURDOCH, C. E., GRIEVE, D. J., CAVE, A. C., LOOI, Y. H. & SHAH, A. M. (2006) NADPH oxidase and heart failure. *Curr Opin Pharmacol*, 6, 148-53.
- MURIEL, P., CASTANEDA, G., ORTEGA, M. & NOEL, F. (2003) Insights into the mechanism of erythrocyte Na⁺/K⁺-ATPase inhibition by nitric oxide and peroxy nitrite anion. *J Appl Toxicol*, 23, 275-8.
- MURIEL P, S. G. (2000) Nitric oxide and peroxy nitrite anion modulate liver plasma membrane fluidity and Na⁺/K⁺-ATPase activity. *Nitric Oxide*, 4, 333-342.
- NAKAO, M. & GADSBY, D. C. (1989) [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 539-65.
- NEUBAUER, S. (2007) The failing heart--an engine out of fuel. *N Engl J Med*, 356, 1140-51.
- OHARA, Y., PETERSON, T. E. & HARRISON, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*, 91, 2546-51.
- PACKER, M., CARVER, J. R., RODEHEFFER, R. J., IVANHOE, R. J., DIBIANCO, R., ZELDIS, S. M., HENDRIX, G. H., BOMMER, W. J., ELKAYAM, U., KUKIN, M. L. & ET AL. (1991) Effect of oral milrinone on

- mortality in severe chronic heart failure. The PROMISE Study Research Group. *N Engl J Med*, 325, 1468-75.
- PARK, J. W., HOYAL, C. R., BENNA, J. E. & BABIOR, B. M. (1997) Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(PHOX) during oxidase activation. *J Biol Chem*, 272, 11035-43.
- PELUFFO, R. D., ARGUELLO, J. M. & BERLIN, J. R. (2000) The role of Na,K-ATPase alpha subunit serine 775 and glutamate 779 in determining the extracellular K⁺ and membrane potential-dependent properties of the Na,K-pump. *J Gen Physiol*, 116, 47-59.
- PELUFFO, R. D., HARA, Y. & BERLIN, J. R. (2004) Quaternary organic amines inhibit Na,K pump current in a voltage-dependent manner: direct evidence of an extracellular access channel in the Na,K-ATPase. *J Gen Physiol*, 123, 249-63.
- PETRUSHANKO, I., BOGDANOV, N., BULYGINA, E., GREINACHER, B., LEINSOO, T., BOLDYREV, A., GASSMANN, M. & BOGDANOVA, A. (2006) Na-K-ATPase in rat cerebellar granule cells is redox sensitive. *Am J Physiol Regul Integr Comp Physiol*, 290, R916-25.
- PIESKE B, MAIER LS, PIACENTINO V 3RD, WEISSER J, HASENFUSS G & HOUSER S (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*, 106, 447-453.
- PIESKE, B. & HOUSER, S. R. (2003) [Na⁺]_i handling in the failing human heart. *Cardiovasc Res*, 57, 874-86.
- PIESKE, B., HOUSER, S. R., HASENFUSS, G. & BERS, D. M. (2003) Sodium and the heart: a hidden key factor in cardiac regulation. *Cardiovasc Res*, 57, 871-2.
- PIESKE, B., MAIER, L. S., PIACENTINO, V., 3RD, WEISSER, J., HASENFUSS, G. & HOUSER, S. (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*, 106, 447-53.
- PLUMB, R. D., EL-SHERBEENY, N. A., DIXON, L. J., HUGHES, S. M., DEVINE, A. B., LEAHEY, W. J. & MCVEIGH, G. E. (2005) NAD(P)H-dependent superoxide production in platelets: the role of angiotensin II and protein kinase C. *Clin Biochem*, 38, 607-13.
- POGWIZD SM, SIPIDO KR, VERDONCK F & BERS DM (2003) Intracellular Na in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. *Cardiovasc Res*, 57, 887-896.
- RAJU, S. V., BAROUCH, L. A. & HARE, J. M. (2005) Nitric oxide and oxidative stress in cardiovascular aging. *Sci Aging Knowledge Environ*, 2005, re4.
- RAKOWSKI, R. F., BEZANILLA, F., DE WEER, P., GADSBY, D. C., HOLMGREN, M. & WAGG, J. (1997a) Charge translocation by the Na/K pump. *Ann N Y Acad Sci*, 834, 231-43.
- RAKOWSKI, R. F., GADSBY, D. C. & DE WEER, P. (1997b) Voltage dependence of the Na/K pump. *J Membr Biol*, 155, 105-12.
- RAO, G. N., SARDET, C., POUYSSEGUR, J. & BERK, B. C. (1990) Differential regulation of Na⁺/H⁺ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J Biol Chem*, 265, 19393-6.

- REY, F. E., CIFUENTES, M. E., KIARASH, A., QUINN, M. T. & PAGANO, P. J. (2001) Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O₂(-) and systolic blood pressure in mice. *Circ Res*, 89, 408-14.
- ROMERO-ALVIRA, D. & ROCHE, E. (1996) High blood pressure, oxygen radicals and antioxidants: etiological relationships. *Med Hypotheses*, 46, 414-20.
- RUSSELL, J. M. (2000) Sodium-potassium-chloride cotransport. *Physiol Rev*, 80, 211-76.
- RYBIN, V. O., XU, X. & STEINBERG, S. F. (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae : stimulation of local protein phosphorylation. *Circ Res*, 84, 980-8.
- SAGAR, A. & RAKOWSKI, R. F. (1994) Access channel model for the voltage dependence of the forward-running Na⁺/K⁺ pump. *J Gen Physiol*, 103, 869-93.
- SATO, A., YAGIHARA, N., KODAMA, M., MITSUMA, W., TACHIKAWA, H., ITO, M., HANAWA, H. & AIZAWA, Y. Takotsubo cardiomyopathy after delivery in an oestrogen-deficient patient. *Int J Cardiol*, 149, e78-9.
- SATO, T., KAMATA, Y., IRIFUNE, M. & NISHIKAWA, T. (1997) Inhibitory effect of several nitric oxide-generating compounds on purified Na⁺,K⁽⁺⁾-ATPase activity from porcine cerebral cortex. *J Neurochem*, 68, 1312-8.
- SEDDON, M., LOOI, Y. H. & SHAH, A. M. (2007) Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart*, 93, 903-7.
- SHAH, A. M. & MACCARTHY, P. A. (2000) Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther*, 86, 49-86.
- SHAINSKAYA, A., SCHNEEBERGER, A., APELL, H. J. & KARLISH, S. J. (2000) Entrance port for Na⁽⁺⁾ and K⁽⁺⁾ ions on Na⁽⁺⁾,K⁽⁺⁾-ATPase in the cytoplasmic loop between trans-membrane segments M6 and M7 of the alpha subunit. Proximity Of the cytoplasmic segment of the beta subunit. *J Biol Chem*, 275, 2019-28.
- SHAMRAJ, O. I. & LINGREL, J. B. (1994) A putative fourth Na⁺,K⁽⁺⁾-ATPase alpha-subunit gene is expressed in testis. *Proc Natl Acad Sci U S A*, 91, 12952-6.
- SHIMON, M. B., GOLDSHLEGER, R. & KARLISH, S. J. (1998) Specific Cu²⁺-catalyzed oxidative cleavage of Na,K-ATPase at the extracellular surface. *J Biol Chem*, 273, 34190-5.
- SHINODA, T., OGAWA, H., CORNELIUS, F. & TOYOSHIMA, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature*, 459, 446-50.
- SILVERMAN, B. Z., FULLER, W., EATON, P., DENG, J., MOORMAN, J. R., CHEUNG, J. Y., JAMES, A. F. & SHATTOCK, M. J. (2005) Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. *Cardiovasc Res*, 65, 93-103.
- SIRAGY, H. M. & CAREY, R. M. (1997) The subtype 2 (AT₂) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*, 100, 264-9.
- SKOU, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta*, 23, 394-401.

- SKOU, J. C. (1998) Nobel Lecture. The identification of the sodium pump. *Biosci Rep*, 18, 155-69.
- SOMERS, M. J., MAVROMATIS, K., GALIS, Z. S. & HARRISON, D. G. (2000) Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation*, 101, 1722-8.
- STADTMAN, E. R. & LEVINE, R. L. (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, 25, 207-18.
- STANTON, B. A. & KAISLING, B. (1989) Regulation of renal ion transport and cell growth by sodium. *Am J Physiol*, 257, F1-10.
- STOLK, J., HILTERMANN, T. J., DIJKMAN, J. H. & VERHOEVEN, A. J. (1994) Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*, 11, 95-102.
- STUEHR, D. J., CHO, H. J., KWON, N. S., WEISE, M. F. & NATHAN, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, 88, 7773-7.
- SWEADNER, K. J. (2005) Phospholemman: a new force in cardiac contractility. *Circ Res*, 97, 510-1.
- SWEADNER, K. J., ARYSTARKHOVA, E., DONNET, C. & WETZEL, R. K. (2003) FXYD proteins as regulators of the Na,K-ATPase in the kidney. *Ann N Y Acad Sci*, 986, 382-7.
- SWEADNER, K. J. & FESCHENKO, M. S. (2001) Predicted location and limited accessibility of protein kinase A phosphorylation site on Na-K-ATPase. *Am J Physiol Cell Physiol*, 280, C1017-26.
- SWEADNER, K. J. & RAEL, E. (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics*, 68, 41-56.
- SWEDBERG, K., ENEROTH, P., KJEKSHUS, J. & WILHELMSEN, L. (1990) Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group. *Circulation*, 82, 1730-6.
- SYDOW, K. & MUNZEL, T. (2003) ADMA and oxidative stress. *Atheroscler Suppl*, 4, 41-51.
- SZABO, C. (2003) Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett*, 140-141, 105-12.
- TAMURA, N., KONISHI, S., IWAKI, S., KIMURA-SOMEYA, T., NADA, S. & YAMAGUCHI, A. (2001) Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J Biol Chem*, 276, 20330-9.
- TAUB, M., BORSICK, M., GEISEL, J., MATLHAGELA, K., RAJKHOWA, T. & ALLEN, C. (2004) Regulation of the Na,K-ATPase in MDCK cells by prostaglandin E1: a role for calcium as well as cAMP. *Exp Cell Res*, 299, 1-14.
- TEN HOVE, M., CHAN, S., LYGATE, C., MONFARED, M., BOEHM, E., HULBERT, K., WATKINS, H., CLARKE, K. & NEUBAUER, S. (2005) Mechanisms of creatine depletion in chronically failing rat heart. *J Mol Cell Cardiol*, 38, 309-13.

- THERIEN, A. G. & BLOSTEIN, R. (1999) K(+)/Na(+) antagonism at cytoplasmic sites of Na(+)-K(+)-ATPase: a tissue-specific mechanism of sodium pump regulation. *Am J Physiol*, 277, C891-8.
- THERIEN, A. G. & BLOSTEIN, R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*, 279, C541-66.
- THERIEN AG, B. R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol.* , 279, 541-66.
- TODA, N., AYAJIKI, K. & OKAMURA, T. (2007) Interaction of endothelial nitric oxide and angiotensin in the circulation. *Pharmacol Rev*, 59, 54-87.
- TOURNEUR, Y., MITRA, R., MORAD, M. & ROUGIER, O. (1987) Activation properties of the inward-rectifying potassium channel on mammalian heart cells. *J Membr Biol*, 97, 127-35.
- TSUTSUI, H. (2004) Novel Pathophysiological Insight and Treatment Strategies for Heart Failure. *Circ J*, 68, 1095-1103.
- TURKO, I. V. & MURAD, F. (2002) Protein nitration in cardiovascular diseases. *Pharmacol Rev*, 54, 619-34.
- VARELA, M., HERRERA, M. & GARVIN, J. L. (2004) Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on O₂- and is diminished by a high-salt diet. *Am J Physiol Renal Physiol*, 287, F224-30.
- VASQUEZ-VIVAR, J., MARTASEK, P., WHITSETT, J., JOSEPH, J. & KALYANARAMAN, B. (2002) The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J*, 362, 733-9.
- VAUGHAN-JONES, R. D., EISNER, D. A. & LEDERER, W. J. (1985) The effects of intracellular Na on contraction and intracellular pH in mammalian cardiac muscle. *Adv Myocardiol*, 5, 313-30.
- VIGNERY, A., WANG, F., QIAN, H. Y., BENZ, E. J., JR. & GILMORE-HEBERT, M. (1991) Detection of the Na(+)-K(+)-ATPase alpha 3-isoform in multinucleated macrophages. *Am J Physiol*, 260, F704-9.
- VILA-PETROFF, M. G., YOUNES, A., EGAN, J., LAKATTA, E. G. & SOLLOTT, S. J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res*, 84, 1020-31.
- VUKOSAVLJEVIC, N., JARON, D., BARBEE, K. A. & BUERK, D. G. (2006) Quantifying the L-arginine paradox in vivo. *Microvasc Res*, 71, 48-54.
- WANG, F., CHEN, C. C., MAO, B. Y., MAO, Q., ZHANG, Y. K., LEI, D. & XU, R. X. (2004) [Effect of L-arginine on the changes of cerebral microcirculation following craniocerebral missile wound at early stage in cats]. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 35, 402-5.
- WANG, Y., GAO, J., MATHIAS, R. T., COHEN, I. S., SUN, X. & BALDO, G. J. (1998) alpha-Adrenergic effects on Na⁺-K⁺ pump current in guinea-pig ventricular myocytes. *J Physiol*, 509 (Pt 1), 117-28.
- WARNHOLTZ, A., NICKENIG, G., SCHULZ, E., MACHARZINA, R., BRASEN, J. H., SKATCHKOV, M., HEITZER, T., STASCH, J. P., GRIENDLING, K. K., HARRISON, D. G., BOHM, M., MEINERTZ, T. & MUNZEL, T. (1999) Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*, 99, 2027-33.

- WENZEL-SEIFERT, K., LIU, H. Y. & SEIFERT, R. (2002) Similarities and differences in the coupling of human beta1- and beta2-adrenoceptors to Gs(alpha) splice variants. *Biochem Pharmacol*, 64, 9-20.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009a) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009b) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., HAMILTON, E. J., GARCIA, A., WANG, D., CHIA, K. K. M., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) Opposing effects of coupled and uncoupled NOS activity on the Na⁺-K⁺ pump in cardiac myocytes. *Am J Physiol Cell Physiol*, 294, C572-8.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2010) Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WHITE, C. N. W., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009c) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008a) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation. *Am J Physiol Cell Physiol*, 294, C1067-73.
- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., FIGTREE, G. A. & RASMUSSEN, H. H. (2008b) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C coupled NOS activation *Am J Physiol (Cell Physiol)*, 294, C1067-73.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005a) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*, 565, 815-25.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005b) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*.
- WILLIAMS, H. C. & GRIENDLING, K. K. (2007) NADPH oxidase inhibitors: new antihypertensive agents? *J Cardiovasc Pharmacol*, 50, 9-16.
- WINTERBOURN, C. C. & HAMPTON, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med*, 45, 549-61.

- XIE Z, KOMETIANI P, LIU J, LI J, SHAPIRO JI & ASKARI A (1999) Intracellular reactive oxygen species mediate the linkage of Na⁺/K⁺-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem.*, 274, 19323-19328.
- YANG, B. & RIZZO, V. (2007) TNF- α potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*, 292, H954-962.
- YAO, L., FAN, P., JIANG, Z., GORDON, A., MOCHLY-ROSEN, D. & DIAMOND, I. (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol*, 73, 1105-12.
- ZAUGG, M., LUCCHINETTI, E., UECKER, M., PASCH, T. & SCHAUB, M. C. (2003) Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth*, 91, 551-65.
- ZHANG, C., IMAM, S. Z., ALI, S. F. & MAYEUX, P. R. (2002) Peroxynitrite and the regulation of Na⁽⁺⁾,K⁽⁺⁾-ATPase activity by angiotensin II in the rat proximal tubule. *Nitric Oxide*, 7, 30-5.
- ZHAO, X., XU, B., BHATTACHARJEE, A., OLDFIELD, C. M., WIENTJES, F. B., FELDMAN, G. M. & CATHCART, M. K. (2005) Protein kinase Cdelta regulates p67phox phosphorylation in human monocytes. *J Leukoc Biol*, 77, 414-20.
- (1990) Xamoterol in severe heart failure. The Xamoterol in Severe Heart Failure Study Group. *Lancet*, 336, 1-6.
- ABEL, E. D. (2004) Glucose transport in the heart. *Front Biosci*, 9, 201-15.
- ADACHI, T., WEISBROD, R. M., PIMENTEL, D. R., YING, J., SHAROV, V. S., SCHONEICH, C. & COHEN, R. A. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med*, 10, 1200-7.
- ALDERTON WK, COOPER CE & KNOWLES RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J.*, 357, 593-615.
- ANVERSA, P., LERI, A., BELTRAMI, C. A., GUERRA, S. & KAJSTURA, J. (1998) Myocyte death and growth in the failing heart. *Lab Invest*, 78, 767-86.
- APELL, H. J. & KARLISH, S. J. (2001) Functional properties of Na,K-ATPase, and their structural implications, as detected with biophysical techniques. *J Membr Biol*, 180, 1-9.
- BABIOR, B. M. (2000) The NADPH oxidase of endothelial cells. *IUBMB Life*, 50, 267-9.
- BAHINSKI, A., NAIRN, A. C., GREENGARD, P. & GADSBY, D. C. (1989) Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*, 340, 718-21.
- BALLIGAND, J. L. (2000) [Beta 3 adrenergic receptor: physiologic role and potential therapeutic applications]. *Bull Mem Acad R Med Belg*, 155, 311-7; discussion 317-9.
- BALLIGAND, J. L., UNGUREANU-LONGROIS, D., SIMMONS, W. W., PIMENTAL, D., MALINSKI, T. A., KAPTURCZAK, M., TAHA, Z.,

- LOWENSTEIN, C. J., DAVIDOFF, A. J., KELLY, R. A. & ET AL. (1994) Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem*, 269, 27580-8.
- BAROUCH, L. A., HARRISON, R. W., SKAF, M. W., ROSAS, G. O., CAPPOLA, T. P., KOBEISSI, Z. A., HOBAL, I. A., LEMMON, C. A., BURNETT, A. L., O'ROURKE, B., RODRIGUEZ, E. R., HUANG, P. L., LIMA, J. A., BERKOWITZ, D. E. & HARE, J. M. (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature*, 416, 337-9.
- BARWE, S. P., KIM, S., RAJASEKARAN, S. A., BOWIE, J. U. & RAJASEKARAN, A. K. (2007) Janus model of the Na,K-ATPase beta-subunit transmembrane domain: distinct faces mediate alpha/beta assembly and beta-beta homo-oligomerization. *J Mol Biol*, 365, 706-14.
- BEDARD, K. & KRAUSE, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*, 87, 245-313.
- BERK, B. C., ELDER, E. & MITSUKA, M. (1990) Hypertrophy and hyperplasia cause differing effects on vascular smooth muscle cell Na⁺/H⁺ exchange and intracellular pH. *J Biol Chem*, 265, 19632-7.
- BERS, D. M. (2001) *Excitation-Contraction Coupling and Cardiac Contractile Force* Dordrecht, The Netherlands, Kluwer Academic Press:427.
- BERS, D. M., BARRY, W. H. & DESPA, S. (2003) Intracellular Na⁺ regulation in cardiac myocytes. *Cardiovasc Res*, 57, 897-912.
- BIBERT, S., ROY, S., SCHAER, D., HORISBERGER, J. D. & GEERING, K. (2008) Phosphorylation of phospholemman (FXD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *J Biol Chem*, 283, 476-86.
- BLANCO, G., SANCHEZ, G., MELTON, R. J., TOURTELLOTTE, W. G. & MERCER, R. W. (2000) The alpha4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *J Histochem Cytochem*, 48, 1023-32.
- BOLDYREV, A. A., BULYGINA, E. R., KRAMARENKO, G. G. & VANIN, A. F. (1997) Effect of nitroso compounds on Na/K-ATPase. *Biochim Biophys Acta*, 1321, 243-51.
- BORGES, V. C., ROCHA, J. B. & NOGUEIRA, C. W. (2005) Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. *Toxicology*, 215, 191-7.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001a) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na(+)-K(+) pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001b) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na⁺-K⁺ pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., GRAY, D. F., MIHAILIDOU, A. S. & RASMUSSEN, H. H. (1999) Angiotensin regulates the selectivity of the Na⁺-K⁺ pump for intracellular Na⁺. *Am J Physiol*, 277, C461-8.

- BUHAGIAR, K. A., HANSEN, P. S., KONG, B. Y., CLARKE, R. J., FERNANDES, C. & RASMUSSEN, H. H. (2004) Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes. *Am J Physiol Cell Physiol*, 286, C398-405.
- BUNDGAARD H, G. A., HAMILTON EJ, WHITE CN, RASMUSSEN HH (2006) Beta-3 receptors mediate adrenergic stimulation of the sarcolemmal Na⁺-K⁺ pump. *Biophys J suppl*, 556a.
- BUTLER, R., MORRIS, A. D., BELCH, J. J., HILL, A. & STRUTHERS, A. D. (2000) Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension*, 35, 746-51.
- CAI, H., GRIENDLING, K. K. & HARRISON, D. G. (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*, 24, 471-8.
- CAI, H. & HARRISON, D. G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*, 87, 840-4.
- CAMBIER, J. C., NEWELL, M. K., JUSTEMENT, L. B., MCGUIRE, J. C., LEACH, K. L. & CHEN, Z. Z. (1987) Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature*, 327, 629-32.
- CAPPOLA, T. P., KASS, D. A., NELSON, G. S., BERGER, R. D., ROSAS, G. O., KOBEISSI, Z. A., MARBAN, E. & HARE, J. M. (2001) Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation*, 104, 2407-11.
- CHARPENTIER, F., LEGATO, M. J., STEINBERG, S. F., COHEN, I. S. & ROSEN, M. R. (1996) Beta-adrenergic modulation of Na-K pump activity in young and adult canine cardiac Purkinje fibers. *Am J Physiol*, 271, H706-12.
- CHENG, X. J., HOOG, J. O., NAIRN, A. C., GREENGARD, P. & APERIA, A. (1997) Regulation of rat Na(+)-K(+)-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am J Physiol*, 273, C1981-6.
- CLAUSEN, T., VAN HARDEVELD, C. & EVERTS, M. E. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev*, 71, 733-74.
- CLAVREUL, N., BACHSCHMID, M. M., HOU, X., SHI, C., IDRIZOVIC, A., IDO, Y., PIMENTEL, D. & COHEN, R. A. (2006) S-glutathiolation of p21ras by peroxynitrite mediates endothelial insulin resistance caused by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol*, 26, 2454-61.
- CLOSS, E. I., SCHELD, J. S., SHARAFI, M. & FORSTERMANN, U. (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol*, 57, 68-74.
- COHEN, R. A. & ADACHI, T. (2006) Nitric-oxide-induced vasodilatation: regulation by physiologic s-glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc Med*, 16, 109-14.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003a) Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci*, 18, 119-24.

- CORNELIUS, F. & MAHMMOUD, Y. A. (2003b) Themes in ion pump regulation. *Ann N Y Acad Sci*, 986, 579-86.
- CORNELIUS, F., MAHMMOUD, Y. A. & CHRISTENSEN, H. R. (2001) Modulation of Na,K-ATPase by associated small transmembrane regulatory proteins and by lipids. *J Bioenerg Biomembr*, 33, 415-23.
- DAIBER, A., ZOU, M. H., BACHSCHMID, M. & ULLRICH, V. (2000) Ebselen as a peroxynitrite scavenger in vitro and ex vivo. *Biochem Pharmacol*, 59, 153-60.
- DE GASPARO, M. & SIRAGY, H. M. (1999) The AT2 receptor: fact, fancy and fantasy. *Regul Pept*, 81, 11-24.
- DESPA, S., BOSSUYT, J., HAN, F., GINSBURG, K. S., JIA, L. G., KUTCHAI, H., TUCKER, A. L. & BERS, D. M. (2005) Phospholemman-phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. *Circ Res*, 97, 252-9.
- DESPA, S., ISLAM, M. A., WEBER, C. R., POGWIZD, S. M. & BERS, D. M. (2002) Intracellular Na(+) concentration is elevated in heart failure but Na/K pump function is unchanged. *Circulation*, 105, 2543-8.
- DOEGE, H., BOCIANSKI, A., SCHEEPERS, A., AXER, H., ECKEL, J., JOOST, H. G. & SCHURMANN, A. (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J*, 359, 443-9.
- DUDZINSKI, D. M., IGARASHI, J., GREIF, D. & MICHEL, T. (2006) The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol*, 46, 235-76.
- EKELUND, U. E., HARRISON, R. W., SHOKEK, O., THAKKAR, R. N., TUNIN, R. S., SENZAKI, H., KASS, D. A., MARBAN, E. & HARE, J. M. (1999) Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circ Res*, 85, 437-45.
- ELLIS, D. Z., RABE, J. & SWEADNER, K. J. (2003) Global loss of Na,K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci*, 23, 43-51.
- ERLENKAMP, S., GLITSCH, H. G. & KOCKSKAMPER, J. (2002) Dual regulation of cardiac Na⁺-K⁺ pumps and CFTR Cl⁻ channels by protein kinases A and C. *Pflugers Arch*, 444, 251-62.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009a) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009b) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FLIEGEL, L. (2008) Molecular biology of the myocardial Na⁺/H⁺ exchanger. *J Mol Cell Cardiol*, 44, 228-37.
- FLIEGEL, L. & FROHLICH, O. (1993) The Na⁺/H⁺ exchanger: an update on structure, regulation and cardiac physiology. *Biochem J*, 296 (Pt 2), 273-85.

- FOLLMER, C. H., TEN EICK, R. E. & YEH, J. Z. (1987) Sodium current kinetics in cat atrial myocytes. *J Physiol*, 384, 169-97.
- FORBUSH, B., 3RD, KAPLAN, J. H. & HOFFMAN, J. F. (1978) Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry*, 17, 3667-76.
- FRIEBE, A. & KOESLING, D. (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res*, 93, 96-105.
- FULLER, W., EATON, P., BELL, J. R. & SHATTOCK, M. J. (2004) Ischemia-induced phosphorylation of phospholemman directly activates rat cardiac Na/K-ATPase. *Faseb J*, 18, 197-9.
- FULLER, W., HOWIE, J., MCLATCHIE, L. M., WEBER, R. J., HASTIE, C. J., BURNES, K., PAVLOVIC, D. & SHATTOCK, M. J. (2009) FXYP1 phosphorylation in vitro and in adult rat cardiac myocytes: threonine 69 is a novel substrate for protein kinase C. *Am J Physiol Cell Physiol*, 296, C1346-55.
- GADSBY, D. C. (2007) Structural biology: ion pumps made crystal clear. *Nature*, 450, 957-9.
- GADSBY, D. C., KIMURA, J. & NOMA, A. (1985) Voltage dependence of Na/K pump current in isolated heart cells. *Nature*, 315, 63-5.
- GADSBY, D. C. & NAKAO, M. (1989) Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 511-37.
- GADSBY, D. C., NAKAO, M. & BAHINSKI, A. (1989) Voltage dependence of transient and steady-state Na/K pump currents in myocytes. *Mol Cell Biochem*, 89, 141-6.
- GALLOGLY, M. M. & MIEYAL, J. J. (2007) Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr Opin Pharmacol*, 7, 381-91.
- GAO, J., COHEN, I. S., MATHIAS, R. T. & BALDO, G. J. (1998) The inhibitory effect of beta-stimulation on the Na/K pump current in guinea pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. *Pflugers Arch*, 435, 479-84.
- GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1992) Isoprenaline, Ca²⁺ and the Na(+)-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 449, 689-704.
- GAO, J., MATHIAS, R. T., COHEN, I. S., WANG, Y., SUN, X. & BALDO, G. J. (1999) Activation of PKC increases Na⁺-K⁺ pump current in ventricular myocytes from guinea pig heart. *Pflugers Arch*, 437, 643-51.
- GARCIA, A., BUNDGAARD, H., HAMILTON, E., LIU, C., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) beta-3 adrenergic stimulation of the Na⁺-K⁺ pump is mediated by NO-dependent deglutathionylation of the beta-1 pump subunit. *12th International ATPase Conference*. Aarhus.
- GARTY, H. & KARLISH, S. J. (2006) Role of FXYP proteins in ion transport. *Annu Rev Physiol*, 68, 431-59.
- GEERING, K. (2001) The functional role of beta subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr*, 33, 425-38.

- GHEZZI, P. (2005) Regulation of protein function by glutathionylation. *Free Radic Res*, 39, 573-80.
- GLITSCH, H. G. (2001) Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev*, 81, 1791-826.
- GOLIGORSKY, M. S., CHEN, J. & BRODSKY, S. (2001) Workshop: endothelial cell dysfunction leading to diabetic nephropathy : focus on nitric oxide. *Hypertension*, 37, 744-8.
- GOLIGORSKY, M. S. & GROSS, S. S. (2001) The ins and outs of endothelial dysfunction: much ado about NO-thing. *Drug News Perspect*, 14, 133-42.
- GRIENGLING, K. K., MINIERI, C. A., OLLERENSHAW, J. D. & ALEXANDER, R. W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*, 74, 1141-8.
- GRIENGLING, K. K., SORESCU, D. & USHIO-FUKAI, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*, 86, 494-501.
- GUIMBAL, C. & KILIMANN, M. W. (1993) A Na(+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem*, 268, 8418-21.
- GUTHIKONDA, S., SINKEY, C., BARENZ, T. & HAYNES, W. G. (2003) Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers. *Circulation*, 107, 416-21.
- HAN, F., BOSSUYT, J., DESPA, S., TUCKER, A. L. & BERS, D. M. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na⁺/K⁺ pump function in cardiac myocytes. *Circ Res*, 99, 1376-83.
- HAN, J., KIM, N., KIM, E., HO, W. K. & EARM, Y. E. (2001) Modulation of ATP-sensitive potassium channels by cGMP-dependent protein kinase in rabbit ventricular myocytes. *J Biol Chem*, 276, 22140-7.
- HANSEN, P. S., BUHAGIAR, K. A., KONG, B. Y., CLARKE, R. J., GRAY, D. F. & RASMUSSEN, H. H. (2002) Dependence of Na⁺-K⁺ pump current-voltage relationship on intracellular Na⁺, K⁺, and Cs⁺ in rabbit cardiac myocytes. *Am J Physiol Cell Physiol*, 283, C1511-21.
- HANSEN, P. S., CLARKE, R. J., BUHAGIAR, K. A., HAMILTON, E., GARCIA, A., WHITE, C. & RASMUSSEN, H. H. (2007) Alloxan-induced diabetes reduces sarcolemmal Na⁺-K⁺ pump function in rabbit ventricular myocytes. *Am J Physiol Cell Physiol*, 292, C1070-1077.
- HARADA, K., LIN, H., ENDO, Y., FUJISHIRO, N., SAKAMOTO, Y. & INOUE, M. (2006) Subunit composition and role of Na⁺,K⁺-ATPases in ventricular myocytes. *J Physiol Sci*, 56, 113-21.
- HARRISON, D. G. (1997) Endothelial function and oxidant stress. *Clin Cardiol*, 20, II-11-7.
- HASLER, U., CRAMBERT, G., HORISBERGER, J. D. & GEERING, K. (2001) Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. *J Biol Chem*, 276, 16356-64.
- HELIN, P. & LORENZEN, I. (1976) Seasonal variations in the susceptibility of the aortic wall to atherosclerosis. Biochemical studies of glycosaminoglycans and collagen of rabbit atherosclerosis. *Atherosclerosis*, 24, 259-66.

- HERRERA, V. L., EMANUEL, J. R., RUIZ-OPAZO, N., LEVENSON, R. & NADAL-GINARD, B. (1987) Three differentially expressed Na,K-ATPase alpha subunit isoforms: structural and functional implications. *J Cell Biol*, 105, 1855-65.
- HESS, D. T., MATSUMOTO, A., KIM, S. O., MARSHALL, H. E. & STAMLER, J. S. (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*, 6, 150-66.
- HEVEL, J. M. & MARLETTA, M. A. (1992) Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry*, 31, 7160-5.
- HINGTGEN, S. D., TIAN, X., YANG, J., DUNLAY, S. M., PEEK, A. S., WU, Y., SHARMA, R. V., ENGELHARDT, J. F. & DAVISSON, R. L. (2006) Nox2-containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. *Physiol Genomics*, 26, 180-91.
- HOFFMANN, C., LEITZ, M. R., OBERDORF-MAASS, S., LOHSE, M. J. & KLOTZ, K. N. (2004) Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*, 369, 151-9.
- HOOL LC, GRAY DF, ROBINSON BG & RASMUSSEN HH (1996) Angiotensin-converting enzyme inhibitors regulate the Na(+)-K+ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-C180.
- HOOL, L. C., GRAY, D. F., ROBINSON, B. G. & RASMUSSEN, H. H. (1996) Angiotensin-converting enzyme inhibitors regulate the Na⁺-K⁺ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-80.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995a) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na(+)-K+ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995b) Angiotensin-converting enzyme inhibition, intracellular Na⁺, and Na⁺-K⁺ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOUSER, S. R. & LAKATTA, E. G. (1999) Function of the cardiac myocyte in the conundrum of end-stage, dilated human heart failure. *Circulation*, 99, 600-4.
- HUANG, B., BLANCO, G., MERCER, R. W., FLEMING, T. & PEPOSE, J. S. (2003) Human corneal endothelial cell expression of Na⁺,K⁺-adenosine triphosphatase isoforms. *Arch Ophthalmol*, 121, 840-5.
- HUANG, N. K., LIN, Y. W., HUANG, C. L., MESSING, R. O. & CHERN, Y. (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. *J Biol Chem*, 276, 13838-46.
- IDE, T., TSUTSUI, H., KINUGAWA, S., UTSUMI, H., KANG, D., HATTORI, N., UCHIDA, K., ARIMURA, K., EGASHIRA, K. & TAKESHITA, A. (1999) Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res*, 85, 357-63.
- IKEHARA, T., YAMAGUCHI, H., HOSOKAWA, K., TAKAHASHI, A. & MIYAMOTO, H. (1993) Kinetic study on the effects of intracellular K⁺ and

- Na⁺ on Na⁺, K⁺, Cl⁻ cotransport of HeLa cells by Rb⁺ influx determination. *J Membr Biol*, 132, 115-24.
- IVANOV, A., ZHAO, H. & MODYANOV, N. N. (2000) Packing of the transmembrane helices of Na,K-ATPase: direct contact between beta-subunit and H8 segment of alpha-subunit revealed by oxidative cross-linking. *Biochemistry*, 39, 9778-85.
- JIA, L. G., DONNET, C., BOGAEV, R. C., BLATT, R. J., MCKINNEY, C. E., DAY, K. H., BERR, S. S., JONES, L. R., MOORMAN, J. R., SWEADNER, K. J. & TUCKER, A. L. (2005) Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice. *Am J Physiol Heart Circ Physiol*, 288, H1982-8.
- JOHNSON, F. & GIULIVI, C. (2005) Superoxide dismutases and their impact upon human health. *Mol Aspects Med*, 26, 340-52.
- KANAI, A. J., MESAROS, S., FINKEL, M. S., ODDIS, C. V., BIRDER, L. A. & MALINSKI, T. (1997) Beta-adrenergic regulation of constitutive nitric oxide synthase in cardiac myocytes. *Am J Physiol*, 273, C1371-7.
- KARLISH, S. J., YATES, D. W. & GLYNN, I. M. (1978) Elementary steps of the (Na⁺ + K⁺)-ATPase mechanism, studied with formycin nucleotides. *Biochim Biophys Acta*, 525, 230-51.
- KLEIN, L., O'CONNOR, C. M., GATTIS, W. A., ZAMPINO, M., DE LUCA, L., VITARELLI, A., FEDELE, F. & GHEORGHIADU, M. (2003) Pharmacologic therapy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations. *Am J Cardiol*, 91, 18F-40F.
- KOCKSKAMPER, J., ERLINKAMP, S. & GLITSCH, H. G. (2000) Activation of the cAMP-protein kinase A pathway facilitates Na⁺ translocation by the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 523 Pt 3, 561-74.
- KOCKSKAMPER, J., SENDHOFF, K., ERLINKAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOCKSKÄMPER, J., SENDHOFF, K., ERLINKAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOJIMA, H., NAKATSUBO, N., KIKUCHI, K., KAWAHARA, S., KIRINO, Y., NAGOSHI, H., HIRATA, Y. & NAGANO, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem*, 70, 2446-53.
- KONG, B. Y. & CLARKE, R. J. (2004) Identification of potential regulatory sites of the Na⁺,K⁺-ATPase by kinetic analysis. *Biochemistry*, 43, 2241-50.
- KOU, R. & MICHEL, T. (2007) Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*, 282, 32719-29.
- KURZ, S. & HARRISON, D. G. (1997) Insulin and the arginine paradox. *J Clin Invest*, 99, 369-70.

- LAMBETH, J. D., KAWAHARA, T. & DIEBOLD, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med*, 43, 319-31.
- LASS, A., SUESSENBACHER, A., WOLKART, G., MAYER, B. & BRUNNER, F. (2002) Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol*, 61, 1081-8.
- LASTRA, G. & MANRIQUE, C. (2007) The expanding role of oxidative stress, renin angiotensin system, and beta-cell dysfunction in the cardiometabolic syndrome and Type 2 diabetes mellitus. *Antioxid Redox Signal*, 9, 943-54.
- LAUGER, P. (1991) Kinetic basis of voltage dependence of the Na,K-pump. *Soc Gen Physiol Ser*, 46, 303-15.
- LEMAY, J., HOU, Y. & DEBLOIS, D. (2000) Evidence that nitric oxide regulates AT1-receptor agonist and antagonist efficacy in rat injured carotid artery. *J Cardiovasc Pharmacol*, 35, 693-9.
- LERMAN, L. O. & LERMAN, A. (2007) All oxidase roads lead to angiotensin, too. *Arterioscler Thromb Vasc Biol*, 27, 703-4.
- LEUNG, P. S. (2004) The peptide hormone angiotensin II: its new functions in tissues and organs. *Curr Protein Pept Sci*, 5, 267-73.
- LI, J. M. & SHAH, A. M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol*, 287, R1014-30.
- LIU, C., FIGTREE, G. A., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F. & RAMUSSEN, H. H. (2008) Oxidative signalling inhibits the cardiac Na⁺-K⁺ pump via glutathionylation. *submitted*.
- LIU, L. & ASKARI, A. (2006) beta-Subunit of cardiac Na⁺-K⁺-ATPase dictates the concentration of the functional enzyme in caveolae. *Am J Physiol Cell Physiol*, 291, C569-78.
- LIU, L., MOHAMMADI, K., AYNAFCHAR, B., WANG, H., LI, D., LIU, J., IVANOV, A. V., XIE, Z. & ASKARI, A. (2003) Role of caveolae in signal-transducing function of cardiac Na⁺/K⁺-ATPase. *Am J Physiol Cell Physiol*, 284, C1550-60.
- LUPFERT, C., GRELL, E., PINTSCHOVIVUS, V., APELL, H. J., CORNELIUS, F. & CLARKE, R. J. (2001) Rate limitation of the Na⁺,K⁺-ATPase pump cycle. *Biophys J*, 81, 2069-81.
- LUTSENKO, S. & KAPLAN, J. H. (1995) Organization of P-type ATPases: significance of structural diversity. *Biochemistry*, 34, 15607-13.
- MAACK, C., KARTES, T., KILTER, H., SCHAFERS, H. J., NICKENIG, G., BOHM, M. & LAUFS, U. (2003) Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation*, 108, 1567-74.
- MALIK, N., CANFIELD, V. A., BECKERS, M. C., GROS, P. & LEVENSON, R. (1996) Identification of the mammalian Na,K-ATPase 3 subunit. *J Biol Chem*, 271, 22754-8.
- MANN, G. E., YUDILEVICH, D. L. & SOBREVIA, L. (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev*, 83, 183-252.

- MARGOLIS, A. S., PORASUPHATANA, S. & ROSEN, G. M. (2000) Role of paraquat in the uncoupling of nitric oxide synthase. *Biochim Biophys Acta*, 1524, 253-7.
- MARTIN-VASALLO, P., DACKOWSKI, W., EMANUEL, J. R. & LEVENSON, R. (1989) Identification of a putative isoform of the Na,K-ATPase beta subunit. Primary structure and tissue-specific expression. *J Biol Chem*, 264, 4613-8.
- MARTINEZ-RUIZ, A. & LAMAS, S. (2007) Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences. *Cardiovasc Res*, 75, 220-8.
- MASSION PB, F. O., DESSY C, BALLIGAND JL (2003) Nitric oxide and cardiac function: ten years after, and continuing. *Circ Res*. . , 93, 388-98.
- MATHERS, J., FRASER, J. A., MCMAHON, M., SAUNDERS, R. D., HAYES, J. D. & MCLELLAN, L. I. (2004) Antioxidant and cytoprotective responses to redox stress. *Biochem Soc Symp*, 157-76.
- MATSUDA, H. & IYANAGI, T. (1999) Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. *Biochim Biophys Acta*, 1473, 345-55.
- MAYER, B., JOHN, M., HEINZEL, B., WERNER, E. R., WACHTER, H., SCHULTZ, G. & BOHME, E. (1991) Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett*, 288, 187-91.
- MERCER, R. W., BIEMESDERFER, D., BLISS, D. P., JR., COLLINS, J. H. & FORBUSH, B., 3RD (1993) Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol*, 121, 579-86.
- MIHAILIDOU, A. S., BUNDGAARD, H., MARDINI, M., HANSEN, P. S., KJELDEN, K. & RASMUSSEN, H. H. (2000) Hyperaldosteronemia in rabbits inhibits the cardiac sarcolemmal Na(+)-K(+) pump. *Circ Res*, 86, 37-42.
- MOHAN, P., BRUTSAERT, D. L., PAULUS, W. J. & SYS, S. U. (1996) Myocardial contractile response to nitric oxide and cGMP. *Circulation*, 93, 1223-9.
- MORGAN T (2003) **Renin, angiotensin, sodium and organ damage.** *Hypertens Res*, 26, 349-54.
- MORTH, J. P., PEDERSEN, B. P., TOUSTRUP-JENSEN, M. S., SORENSEN, T. L., PETERSEN, J., ANDERSEN, J. P., VILSEN, B. & NISSEN, P. (2007) Crystal structure of the sodium-potassium pump. *Nature*, 450, 1043-9.
- MUDD, J. O. & KASS, D. A. (2008) Tackling heart failure in the twenty-first century. *Nature*, 451, 919-28.
- MUNSON, K., LAMBRECHT, N., SHIN, J. M. & SACHS, G. (2000) Analysis of the membrane domain of the gastric H(+)/K(+)-ATPase. *J Exp Biol*, 203, 161-70.
- MURDOCH, C. E., GRIEVE, D. J., CAVE, A. C., LOOI, Y. H. & SHAH, A. M. (2006) NADPH oxidase and heart failure. *Curr Opin Pharmacol*, 6, 148-53.

- MURIEL, P., CASTANEDA, G., ORTEGA, M. & NOEL, F. (2003) Insights into the mechanism of erythrocyte Na⁺/K⁺-ATPase inhibition by nitric oxide and peroxyxynitrite anion. *J Appl Toxicol*, 23, 275-8.
- MURIEL P, S. G. (2000) Nitric oxide and peroxyxynitrite anion modulate liver plasma membrane fluidity and Na⁽⁺⁾/K⁽⁺⁾-ATPase activity. *Nitric Oxide*.2, 4, 333- 342.
- NAKAO, M. & GADSBY, D. C. (1989) [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 539-65.
- NEUBAUER, S. (2007) The failing heart--an engine out of fuel. *N Engl J Med*, 356, 1140-51.
- OHARA, Y., PETERSON, T. E. & HARRISON, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*, 91, 2546-51.
- PACKER, M., CARVER, J. R., RODEHEFFER, R. J., IVANHOE, R. J., DIBIANCO, R., ZELDIS, S. M., HENDRIX, G. H., BOMMER, W. J., ELKAYAM, U., KUKIN, M. L. & ET AL. (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. *N Engl J Med*, 325, 1468-75.
- PARK, J. W., HOYAL, C. R., BENNA, J. E. & BABIOR, B. M. (1997) Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(PHOX) during oxidase activation. *J Biol Chem*, 272, 11035-43.
- PELUFFO, R. D., ARGUELLO, J. M. & BERLIN, J. R. (2000) The role of Na,K-ATPase alpha subunit serine 775 and glutamate 779 in determining the extracellular K⁺ and membrane potential-dependent properties of the Na,K-pump. *J Gen Physiol*, 116, 47-59.
- PELUFFO, R. D., HARA, Y. & BERLIN, J. R. (2004) Quaternary organic amines inhibit Na,K pump current in a voltage-dependent manner: direct evidence of an extracellular access channel in the Na,K-ATPase. *J Gen Physiol*, 123, 249-63.
- PETRUSHANKO, I., BOGDANOV, N., BULYGINA, E., GRENACHER, B., LEINSOO, T., BOLDYREV, A., GASSMANN, M. & BOGDANOVA, A. (2006) Na-K-ATPase in rat cerebellar granule cells is redox sensitive. *Am J Physiol Regul Integr Comp Physiol*, 290, R916-25.
- PIESKE B, MAIER LS, PIACENTINO V 3RD, WEISSER J, HASENFUSS G & HOUSER S (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*., 106, 447-453.
- PIESKE, B. & HOUSER, S. R. (2003) [Na⁺]_i handling in the failing human heart. *Cardiovasc Res*, 57, 874-86.
- PIESKE, B., HOUSER, S. R., HASENFUSS, G. & BERS, D. M. (2003) Sodium and the heart: a hidden key factor in cardiac regulation. *Cardiovasc Res*, 57, 871-2.
- PIESKE, B., MAIER, L. S., PIACENTINO, V., 3RD, WEISSER, J., HASENFUSS, G. & HOUSER, S. (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*, 106, 447-53.
- PLUMB, R. D., EL-SHERBEENY, N. A., DIXON, L. J., HUGHES, S. M., DEVINE, A. B., LEAHEY, W. J. & MCVEIGH, G. E. (2005) NAD(P)H-

- dependent superoxide production in platelets: the role of angiotensin II and protein kinase C. *Clin Biochem*, 38, 607-13.
- POGWIZD SM, SIPIDO KR, VERDONCK F & BERS DM (2003) Intracellular Na in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. *Cardiovasc Res*, 57, 887-896.
- RAJU, S. V., BAROUCH, L. A. & HARE, J. M. (2005) Nitric oxide and oxidative stress in cardiovascular aging. *Sci Aging Knowledge Environ*, 2005, re4.
- RAKOWSKI, R. F., BEZANILLA, F., DE WEER, P., GADSBY, D. C., HOLMGREN, M. & WAGG, J. (1997a) Charge translocation by the Na/K pump. *Ann N Y Acad Sci*, 834, 231-43.
- RAKOWSKI, R. F., GADSBY, D. C. & DE WEER, P. (1997b) Voltage dependence of the Na/K pump. *J Membr Biol*, 155, 105-12.
- RAO, G. N., SARDET, C., POUYSSEGUR, J. & BERK, B. C. (1990) Differential regulation of Na⁺/H⁺ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J Biol Chem*, 265, 19393-6.
- REY, F. E., CIFUENTES, M. E., KIARASH, A., QUINN, M. T. & PAGANO, P. J. (2001) Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O₂(-) and systolic blood pressure in mice. *Circ Res*, 89, 408-14.
- ROMERO-ALVIRA, D. & ROCHE, E. (1996) High blood pressure, oxygen radicals and antioxidants: etiological relationships. *Med Hypotheses*, 46, 414-20.
- RUSSELL, J. M. (2000) Sodium-potassium-chloride cotransport. *Physiol Rev*, 80, 211-76.
- RYBIN, V. O., XU, X. & STEINBERG, S. F. (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae : stimulation of local protein phosphorylation. *Circ Res*, 84, 980-8.
- SAGAR, A. & RAKOWSKI, R. F. (1994) Access channel model for the voltage dependence of the forward-running Na⁺/K⁺ pump. *J Gen Physiol*, 103, 869-93.
- SATO, A., YAGIHARA, N., KODAMA, M., MITSUMA, W., TACHIKAWA, H., ITO, M., HANAWA, H. & AIZAWA, Y. Takotsubo cardiomyopathy after delivery in an oestrogen-deficient patient. *Int J Cardiol*, 149, e78-9.
- SATO, T., KAMATA, Y., IRIFUNE, M. & NISHIKAWA, T. (1997) Inhibitory effect of several nitric oxide-generating compounds on purified Na⁺,K(+) -ATPase activity from porcine cerebral cortex. *J Neurochem*, 68, 1312-8.
- SEDDON, M., LOOI, Y. H. & SHAH, A. M. (2007) Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart*, 93, 903-7.
- SHAH, A. M. & MACCARTHY, P. A. (2000) Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther*, 86, 49-86.
- SHAINSKAYA, A., SCHNEEBERGER, A., APELL, H. J. & KARLISH, S. J. (2000) Entrance port for Na(+) and K(+) ions on Na(+),K(+)-ATPase in the cytoplasmic loop between trans-membrane segments M6 and M7 of the alpha subunit. Proximity Of the cytoplasmic segment of the beta subunit. *J Biol Chem*, 275, 2019-28.
- SHAMRAJ, O. I. & LINGREL, J. B. (1994) A putative fourth Na⁺,K(+) -ATPase alpha-subunit gene is expressed in testis. *Proc Natl Acad Sci U S A*, 91, 12952-6.

- SHIMON, M. B., GOLDSHLEGER, R. & KARLISH, S. J. (1998) Specific Cu²⁺-catalyzed oxidative cleavage of Na,K-ATPase at the extracellular surface. *J Biol Chem*, 273, 34190-5.
- SHINODA, T., OGAWA, H., CORNELIUS, F. & TOYOSHIMA, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature*, 459, 446-50.
- SILVERMAN, B. Z., FULLER, W., EATON, P., DENG, J., MOORMAN, J. R., CHEUNG, J. Y., JAMES, A. F. & SHATTOCK, M. J. (2005) Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. *Cardiovasc Res*, 65, 93-103.
- SIRAGY, H. M. & CAREY, R. M. (1997) The subtype 2 (AT₂) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*, 100, 264-9.
- SKOU, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta*, 23, 394-401.
- SKOU, J. C. (1998) Nobel Lecture. The identification of the sodium pump. *Biosci Rep*, 18, 155-69.
- SOMERS, M. J., MAVROMATIS, K., GALIS, Z. S. & HARRISON, D. G. (2000) Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation*, 101, 1722-8.
- STADTMAN, E. R. & LEVINE, R. L. (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, 25, 207-18.
- STANTON, B. A. & KAISLING, B. (1989) Regulation of renal ion transport and cell growth by sodium. *Am J Physiol*, 257, F1-10.
- STOLK, J., HILTERMANN, T. J., DIJKMAN, J. H. & VERHOEVEN, A. J. (1994) Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*, 11, 95-102.
- STUEHR, D. J., CHO, H. J., KWON, N. S., WEISE, M. F. & NATHAN, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, 88, 7773-7.
- SWEADNER, K. J. (2005) Phospholemman: a new force in cardiac contractility. *Circ Res*, 97, 510-1.
- SWEADNER, K. J., ARYSTARKHOVA, E., DONNET, C. & WETZEL, R. K. (2003) FXYD proteins as regulators of the Na,K-ATPase in the kidney. *Ann N Y Acad Sci*, 986, 382-7.
- SWEADNER, K. J. & FESCHENKO, M. S. (2001) Predicted location and limited accessibility of protein kinase A phosphorylation site on Na-K-ATPase. *Am J Physiol Cell Physiol*, 280, C1017-26.
- SWEADNER, K. J. & RAEL, E. (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics*, 68, 41-56.
- SWEDBERG, K., ENEROTH, P., KJEKSHUS, J. & WILHELMSEN, L. (1990) Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group. *Circulation*, 82, 1730-6.

- SYDOW, K. & MUNZEL, T. (2003) ADMA and oxidative stress. *Atheroscler Suppl*, 4, 41-51.
- SZABO, C. (2003) Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett*, 140-141, 105-12.
- TAMURA, N., KONISHI, S., IWAKI, S., KIMURA-SOMEYA, T., NADA, S. & YAMAGUCHI, A. (2001) Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J Biol Chem*, 276, 20330-9.
- TAUB, M., BORSICK, M., GEISEL, J., MATLHAGELA, K., RAJKHOWA, T. & ALLEN, C. (2004) Regulation of the Na,K-ATPase in MDCK cells by prostaglandin E1: a role for calcium as well as cAMP. *Exp Cell Res*, 299, 1-14.
- TEN HOVE, M., CHAN, S., LYGATE, C., MONFARED, M., BOEHM, E., HULBERT, K., WATKINS, H., CLARKE, K. & NEUBAUER, S. (2005) Mechanisms of creatine depletion in chronically failing rat heart. *J Mol Cell Cardiol*, 38, 309-13.
- THERIEN, A. G. & BLOSTEIN, R. (1999) K(+)/Na(+) antagonism at cytoplasmic sites of Na(+)-K(+)-ATPase: a tissue-specific mechanism of sodium pump regulation. *Am J Physiol*, 277, C891-8.
- THERIEN, A. G. & BLOSTEIN, R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*, 279, C541-66.
- THERIEN AG, B. R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol.* , 279, 541-66.
- TODA, N., AYAJIKI, K. & OKAMURA, T. (2007) Interaction of endothelial nitric oxide and angiotensin in the circulation. *Pharmacol Rev*, 59, 54-87.
- TOURNEUR, Y., MITRA, R., MORAD, M. & ROUGIER, O. (1987) Activation properties of the inward-rectifying potassium channel on mammalian heart cells. *J Membr Biol*, 97, 127-35.
- TSUTSUI, H. (2004) Novel Pathophysiological Insight and Treatment Strategies for Heart Failure. *Circ J*, 68, 1095-1103.
- TURKO, I. V. & MURAD, F. (2002) Protein nitration in cardiovascular diseases. *Pharmacol Rev*, 54, 619-34.
- VARELA, M., HERRERA, M. & GARVIN, J. L. (2004) Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on O₂- and is diminished by a high-salt diet. *Am J Physiol Renal Physiol*, 287, F224-30.
- VASQUEZ-VIVAR, J., MARTASEK, P., WHITSETT, J., JOSEPH, J. & KALYANARAMAN, B. (2002) The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J*, 362, 733-9.
- VAUGHAN-JONES, R. D., EISNER, D. A. & LEDERER, W. J. (1985) The effects of intracellular Na on contraction and intracellular pH in mammalian cardiac muscle. *Adv Myocardiol*, 5, 313-30.
- VIGNERY, A., WANG, F., QIAN, H. Y., BENZ, E. J., JR. & GILMORE-HEBERT, M. (1991) Detection of the Na(+)-K(+)-ATPase alpha 3-isoform in multinucleated macrophages. *Am J Physiol*, 260, F704-9.
- VILA-PETROFF, M. G., YOUNES, A., EGAN, J., LAKATTA, E. G. & SOLLOTT, S. J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res*, 84, 1020-31.

- VUKOSAVLJEVIC, N., JARON, D., BARBEE, K. A. & BUERK, D. G. (2006) Quantifying the L-arginine paradox in vivo. *Microvasc Res*, 71, 48-54.
- WANG, F., CHEN, C. C., MAO, B. Y., MAO, Q., ZHANG, Y. K., LEI, D. & XU, R. X. (2004) [Effect of L-arginine on the changes of cerebral microcirculation following craniocerebral missile wound at early stage in cats]. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 35, 402-5.
- WANG, Y., GAO, J., MATHIAS, R. T., COHEN, I. S., SUN, X. & BALDO, G. J. (1998) alpha-Adrenergic effects on Na⁺-K⁺ pump current in guinea-pig ventricular myocytes. *J Physiol*, 509 (Pt 1), 117-28.
- WARNHOLTZ, A., NICKENIG, G., SCHULZ, E., MACHARZINA, R., BRASEN, J. H., SKATCHKOV, M., HEITZER, T., STASCH, J. P., GRIENDLING, K. K., HARRISON, D. G., BOHM, M., MEINERTZ, T. & MUNZEL, T. (1999) Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*, 99, 2027-33.
- WENZEL-SEIFERT, K., LIU, H. Y. & SEIFERT, R. (2002) Similarities and differences in the coupling of human beta1- and beta2-adrenoceptors to Gs(alpha) splice variants. *Biochem Pharmacol*, 64, 9-20.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009a) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009b) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., HAMILTON, E. J., GARCIA, A., WANG, D., CHIA, K. K. M., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) Opposing effects of coupled and uncoupled NOS activity on the Na⁺-K⁺ pump in cardiac myocytes. *Am J Physiol Cell Physiol*, 294, C572-8.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2010) Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WHITE, C. N. W., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009c) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008a) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation. *Am J Physiol Cell Physiol*, 294, C1067-73.

- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., FIGTREE, G. A. & RASMUSSEN, H. H. (2008b) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C coupled NOS activation *Am J Physiol (Cell Physiol)*, 294, C1067-73.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005a) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*, 565, 815-25.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005b) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*.
- WILLIAMS, H. C. & GRIENDLING, K. K. (2007) NADPH oxidase inhibitors: new antihypertensive agents? *J Cardiovasc Pharmacol*, 50, 9-16.
- WINTERBOURN, C. C. & HAMPTON, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med*, 45, 549-61.
- XIE Z, KOMETIANI P, LIU J, LI J, SHAPIRO JI & ASKARI A (1999) Intracellular reactive oxygen species mediate the linkage of Na⁺/K⁺-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem.*, 274, 19323-19328.
- YANG, B. & RIZZO, V. (2007) TNF- α potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*, 292, H954-962.
- YAO, L., FAN, P., JIANG, Z., GORDON, A., MOCHLY-ROSEN, D. & DIAMOND, I. (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol*, 73, 1105-12.
- ZAUGG, M., LUCCHINETTI, E., UECKER, M., PASCH, T. & SCHAUB, M. C. (2003) Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth*, 91, 551-65.
- ZHANG, C., IMAM, S. Z., ALI, S. F. & MAYEUX, P. R. (2002) Peroxynitrite and the regulation of Na⁽⁺⁾,K⁽⁺⁾-ATPase activity by angiotensin II in the rat proximal tubule. *Nitric Oxide*, 7, 30-5.
- ZHAO, X., XU, B., BHATTACHARJEE, A., OLDFIELD, C. M., WIENTJES, F. B., FELDMAN, G. M. & CATHCART, M. K. (2005) Protein kinase Cdelta regulates p67phox phosphorylation in human monocytes. *J Leukoc Biol*, 77, 414-20.
- (1990) Xamoterol in severe heart failure. The Xamoterol in Severe Heart Failure Study Group. *Lancet*, 336, 1-6.
- ABEL, E. D. (2004) Glucose transport in the heart. *Front Biosci*, 9, 201-15.
- ADACHI, T., WEISBROD, R. M., PIMENTEL, D. R., YING, J., SHAROV, V. S., SCHONEICH, C. & COHEN, R. A. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med*, 10, 1200-7.
- ALDERTON WK, COOPER CE & KNOWLES RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J.*, 357, 593-615.

- ANVERSA, P., LERI, A., BELTRAMI, C. A., GUERRA, S. & KAJSTURA, J. (1998) Myocyte death and growth in the failing heart. *Lab Invest*, 78, 767-86.
- APELL, H. J. & KARLISH, S. J. (2001) Functional properties of Na,K-ATPase, and their structural implications, as detected with biophysical techniques. *J Membr Biol*, 180, 1-9.
- BABIOR, B. M. (2000) The NADPH oxidase of endothelial cells. *IUBMB Life*, 50, 267-9.
- BAHINSKI, A., NAIRN, A. C., GREENGARD, P. & GADSBY, D. C. (1989) Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*, 340, 718-21.
- BALLIGAND, J. L. (2000) [Beta 3 adrenergic receptor: physiologic role and potential therapeutic applications]. *Bull Mem Acad R Med Belg*, 155, 311-7; discussion 317-9.
- BALLIGAND, J. L., UNGUREANU-LONGROIS, D., SIMMONS, W. W., PIMENTAL, D., MALINSKI, T. A., KAPTURCZAK, M., TAHA, Z., LOWENSTEIN, C. J., DAVIDOFF, A. J., KELLY, R. A. & ET AL. (1994) Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem*, 269, 27580-8.
- BAROUCH, L. A., HARRISON, R. W., SKAF, M. W., ROSAS, G. O., CAPPOLA, T. P., KOBEISSI, Z. A., HOBAL, I. A., LEMMON, C. A., BURNETT, A. L., O'ROURKE, B., RODRIGUEZ, E. R., HUANG, P. L., LIMA, J. A., BERKOWITZ, D. E. & HARE, J. M. (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature*, 416, 337-9.
- BARWE, S. P., KIM, S., RAJASEKARAN, S. A., BOWIE, J. U. & RAJASEKARAN, A. K. (2007) Janus model of the Na,K-ATPase beta-subunit transmembrane domain: distinct faces mediate alpha/beta assembly and beta-beta homo-oligomerization. *J Mol Biol*, 365, 706-14.
- BEDARD, K. & KRAUSE, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*, 87, 245-313.
- BERK, B. C., ELDER, E. & MITSUKA, M. (1990) Hypertrophy and hyperplasia cause differing effects on vascular smooth muscle cell Na⁺/H⁺ exchange and intracellular pH. *J Biol Chem*, 265, 19632-7.
- BERS, D. M. (2001) *Excitation-Contraction Coupling and Cardiac Contractile Force* Dordrecht, The Netherlands, Kluwer Academic Press:427.
- BERS, D. M., BARRY, W. H. & DESPA, S. (2003) Intracellular Na⁺ regulation in cardiac myocytes. *Cardiovasc Res*, 57, 897-912.
- BIBERT, S., ROY, S., SCHAER, D., HORISBERGER, J. D. & GEERING, K. (2008) Phosphorylation of phospholemman (FXD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *J Biol Chem*, 283, 476-86.
- BLANCO, G., SANCHEZ, G., MELTON, R. J., TOURTELLOTTE, W. G. & MERCER, R. W. (2000) The alpha4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *J Histochem Cytochem*, 48, 1023-32.

- BOLDYREV, A. A., BULYGINA, E. R., KRAMARENKO, G. G. & VANIN, A. F. (1997) Effect of nitroso compounds on Na/K-ATPase. *Biochim Biophys Acta*, 1321, 243-51.
- BORGES, V. C., ROCHA, J. B. & NOGUEIRA, C. W. (2005) Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. *Toxicology*, 215, 191-7.
- BUHAGIAR, K. A., HANSEN, P. S., BEWICK, N. L. & RASMUSSEN, H. H. (2001) Protein kinase Cepsilon contributes to regulation of the sarcolemmal Na⁺-K⁺ pump. *Am J Physiol Cell Physiol*, 281, C1059-63.
- BUHAGIAR, K. A., HANSEN, P. S., GRAY, D. F., MIHAILIDOU, A. S. & RASMUSSEN, H. H. (1999) Angiotensin regulates the selectivity of the Na⁺-K⁺ pump for intracellular Na⁺. *Am J Physiol*, 277, C461-8.
- BUHAGIAR, K. A., HANSEN, P. S., KONG, B. Y., CLARKE, R. J., FERNANDES, C. & RASMUSSEN, H. H. (2004) Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes. *Am J Physiol Cell Physiol*, 286, C398-405.
- BUNDGAARD H, G. A., HAMILTON EJ, WHITE CN, RASMUSSEN HH (2006) Beta-3 receptors mediate adrenergic stimulation of the sarcolemmal Na⁺-K⁺ pump. *Biophys J suppl*, 556a.
- BUTLER, R., MORRIS, A. D., BELCH, J. J., HILL, A. & STRUTHERS, A. D. (2000) Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension*, 35, 746-51.
- CAI, H., GRIENGLING, K. K. & HARRISON, D. G. (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*, 24, 471-8.
- CAI, H. & HARRISON, D. G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*, 87, 840-4.
- CAMBIER, J. C., NEWELL, M. K., JUSTEMENT, L. B., MCGUIRE, J. C., LEACH, K. L. & CHEN, Z. Z. (1987) Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature*, 327, 629-32.
- CAPPOLA, T. P., KASS, D. A., NELSON, G. S., BERGER, R. D., ROSAS, G. O., KOBEISSI, Z. A., MARBAN, E. & HARE, J. M. (2001) Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation*, 104, 2407-11.
- CHARPENTIER, F., LEGATO, M. J., STEINBERG, S. F., COHEN, I. S. & ROSEN, M. R. (1996) Beta-adrenergic modulation of Na-K pump activity in young and adult canine cardiac Purkinje fibers. *Am J Physiol*, 271, H706-12.
- CHENG, X. J., HOOG, J. O., NAIRN, A. C., GREENGARD, P. & APERIA, A. (1997) Regulation of rat Na(+)-K(+)-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am J Physiol*, 273, C1981-6.
- CLAUSEN, T., VAN HARDEVELD, C. & EVERTS, M. E. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev*, 71, 733-74.
- CLAVREUL, N., BACHSCHMID, M. M., HOU, X., SHI, C., IDRIZOVIC, A., IDO, Y., PIMENTEL, D. & COHEN, R. A. (2006) S-glutathiolation of p21ras by

- peroxynitrite mediates endothelial insulin resistance caused by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol*, 26, 2454-61.
- CLOSS, E. I., SCHELD, J. S., SHARAFI, M. & FORSTERMANN, U. (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol*, 57, 68-74.
- COHEN, R. A. & ADACHI, T. (2006) Nitric-oxide-induced vasodilatation: regulation by physiologic s-glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc Med*, 16, 109-14.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003a) Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci*, 18, 119-24.
- CORNELIUS, F. & MAHMMOUD, Y. A. (2003b) Themes in ion pump regulation. *Ann N Y Acad Sci*, 986, 579-86.
- CORNELIUS, F., MAHMMOUD, Y. A. & CHRISTENSEN, H. R. (2001) Modulation of Na,K-ATPase by associated small transmembrane regulatory proteins and by lipids. *J Bioenerg Biomembr*, 33, 415-23.
- DAIBER, A., ZOU, M. H., BACHSCHMID, M. & ULLRICH, V. (2000) Ebselen as a peroxynitrite scavenger in vitro and ex vivo. *Biochem Pharmacol*, 59, 153-60.
- DE GASPARO, M. & SIRAGY, H. M. (1999) The AT2 receptor: fact, fancy and fantasy. *Regul Pept*, 81, 11-24.
- DESPA, S., BOSSUYT, J., HAN, F., GINSBURG, K. S., JIA, L. G., KUTCHAI, H., TUCKER, A. L. & BERS, D. M. (2005) Phospholemmann-phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. *Circ Res*, 97, 252-9.
- DESPA, S., ISLAM, M. A., WEBER, C. R., POGWIZD, S. M. & BERS, D. M. (2002) Intracellular Na(+) concentration is elevated in heart failure but Na/K pump function is unchanged. *Circulation*, 105, 2543-8.
- DOEGE, H., BOCIANSKI, A., SCHEEPERS, A., AXER, H., ECKEL, J., JOOST, H. G. & SCHURMANN, A. (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J*, 359, 443-9.
- DUDZINSKI, D. M., IGARASHI, J., GREIF, D. & MICHEL, T. (2006) The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol*, 46, 235-76.
- EKELUND, U. E., HARRISON, R. W., SHOKEK, O., THAKKAR, R. N., TUNIN, R. S., SENZAKI, H., KASS, D. A., MARBAN, E. & HARE, J. M. (1999) Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circ Res*, 85, 437-45.
- ELLIS, D. Z., RABE, J. & SWEADNER, K. J. (2003) Global loss of Na,K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci*, 23, 43-51.
- ERLENKAMP, S., GLITSCH, H. G. & KOCKSKAMPER, J. (2002) Dual regulation of cardiac Na⁺-K⁺ pumps and CFTR Cl⁻ channels by protein kinases A and C. *Pflugers Arch*, 444, 251-62.

- FIGTREE, G. A., LIU, C. C., BIBERT, S., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K., CORNELIUS, F., GEERING, K. & RASMUSSEN, H. H. (2009) Reversible oxidative modification: a key mechanism of Na⁺-K⁺ pump regulation. *Circ Res*, 105, 185-93.
- FLIEGEL, L. (2008) Molecular biology of the myocardial Na⁺/H⁺ exchanger. *J Mol Cell Cardiol*, 44, 228-37.
- FLIEGEL, L. & FROHLICH, O. (1993) The Na⁺/H⁺ exchanger: an update on structure, regulation and cardiac physiology. *Biochem J*, 296 (Pt 2), 273-85.
- FOLLMER, C. H., TEN EICK, R. E. & YEH, J. Z. (1987) Sodium current kinetics in cat atrial myocytes. *J Physiol*, 384, 169-97.
- FORBUSH, B., 3RD, KAPLAN, J. H. & HOFFMAN, J. F. (1978) Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry*, 17, 3667-76.
- FRIEBE, A. & KOESLING, D. (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res*, 93, 96-105.
- FULLER, W., EATON, P., BELL, J. R. & SHATTOCK, M. J. (2004) Ischemia-induced phosphorylation of phospholemman directly activates rat cardiac Na/K-ATPase. *Faseb J*, 18, 197-9.
- FULLER, W., HOWIE, J., MCLATCHIE, L. M., WEBER, R. J., HASTIE, C. J., BURNES, K., PAVLOVIC, D. & SHATTOCK, M. J. (2009) FXD1 phosphorylation in vitro and in adult rat cardiac myocytes: threonine 69 is a novel substrate for protein kinase C. *Am J Physiol Cell Physiol*, 296, C1346-55.
- GADSBY, D. C. (2007) Structural biology: ion pumps made crystal clear. *Nature*, 450, 957-9.
- GADSBY, D. C., KIMURA, J. & NOMA, A. (1985) Voltage dependence of Na/K pump current in isolated heart cells. *Nature*, 315, 63-5.
- GADSBY, D. C. & NAKAO, M. (1989) Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 511-37.
- GADSBY, D. C., NAKAO, M. & BAHINSKI, A. (1989) Voltage dependence of transient and steady-state Na/K pump currents in myocytes. *Mol Cell Biochem*, 89, 141-6.
- GALLOGLY, M. M. & MIEYAL, J. J. (2007) Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr Opin Pharmacol*, 7, 381-91.
- GAO, J., COHEN, I. S., MATHIAS, R. T. & BALDO, G. J. (1998) The inhibitory effect of beta-stimulation on the Na/K pump current in guinea pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. *Pflugers Arch*, 435, 479-84.
- GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1992) Isoprenaline, Ca²⁺ and the Na(+)-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 449, 689-704.
- GAO, J., MATHIAS, R. T., COHEN, I. S., WANG, Y., SUN, X. & BALDO, G. J. (1999) Activation of PKC increases Na⁺-K⁺ pump current in ventricular myocytes from guinea pig heart. *Pflugers Arch*, 437, 643-51.

- GARCIA, A., BUNDGAARD, H., HAMILTON, E., LIU, C., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) beta-3 adrenergic stimulation of the Na⁺-K⁺ pump is mediated by NO-dependent deglutathionylation of the beta-1 pump subunit. *12th International ATPase Conference*. Aarhus.
- GARTY, H. & KARLISH, S. J. (2006) Role of FXYD proteins in ion transport. *Annu Rev Physiol*, 68, 431-59.
- GEERING, K. (2001) The functional role of beta subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr*, 33, 425-38.
- GHEZZI, P. (2005) Regulation of protein function by glutathionylation. *Free Radic Res*, 39, 573-80.
- GLITSCH, H. G. (2001) Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev*, 81, 1791-826.
- GOLIGORSKY, M. S., CHEN, J. & BRODSKY, S. (2001) Workshop: endothelial cell dysfunction leading to diabetic nephropathy : focus on nitric oxide. *Hypertension*, 37, 744-8.
- GOLIGORSKY, M. S. & GROSS, S. S. (2001) The ins and outs of endothelial dysfunction: much ado about NO-thing. *Drug News Perspect*, 14, 133-42.
- GRIENGLING, K. K., MINIERI, C. A., OLLERENSHAW, J. D. & ALEXANDER, R. W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*, 74, 1141-8.
- GRIENGLING, K. K., SORESCU, D. & USHIO-FUKAI, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*, 86, 494-501.
- GUIMBAL, C. & KILIMANN, M. W. (1993) A Na⁽⁺⁾-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem*, 268, 8418-21.
- GUTHIKONDA, S., SINKEY, C., BARENZ, T. & HAYNES, W. G. (2003) Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers. *Circulation*, 107, 416-21.
- HAN, F., BOSSUYT, J., DESPA, S., TUCKER, A. L. & BERS, D. M. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na⁺/K⁺ pump function in cardiac myocytes. *Circ Res*, 99, 1376-83.
- HAN, J., KIM, N., KIM, E., HO, W. K. & EARM, Y. E. (2001) Modulation of ATP-sensitive potassium channels by cGMP-dependent protein kinase in rabbit ventricular myocytes. *J Biol Chem*, 276, 22140-7.
- HANSEN, P. S., BUHAGIAR, K. A., KONG, B. Y., CLARKE, R. J., GRAY, D. F. & RASMUSSEN, H. H. (2002) Dependence of Na⁺-K⁺ pump current-voltage relationship on intracellular Na⁺, K⁺, and Cs⁺ in rabbit cardiac myocytes. *Am J Physiol Cell Physiol*, 283, C1511-21.
- HANSEN, P. S., CLARKE, R. J., BUHAGIAR, K. A., HAMILTON, E., GARCIA, A., WHITE, C. & RASMUSSEN, H. H. (2007) Alloxan-induced diabetes reduces sarcolemmal Na⁺-K⁺ pump function in rabbit ventricular myocytes. *Am J Physiol Cell Physiol*, 292, C1070-1077.
- HARADA, K., LIN, H., ENDO, Y., FUJISHIRO, N., SAKAMOTO, Y. & INOUE, M. (2006) Subunit composition and role of Na⁺,K⁺-ATPases in ventricular myocytes. *J Physiol Sci*, 56, 113-21.

- HARRISON, D. G. (1997) Endothelial function and oxidant stress. *Clin Cardiol*, 20, II-11-7.
- HASLER, U., CRAMBERT, G., HORISBERGER, J. D. & GEERING, K. (2001) Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. *J Biol Chem*, 276, 16356-64.
- HELIN, P. & LORENZEN, I. (1976) Seasonal variations in the susceptibility of the aortic wall to atherosclerosis. Biochemical studies of glycosaminoglycans and collagen of rabbit atherosclerosis. *Atherosclerosis*, 24, 259-66.
- HERRERA, V. L., EMANUEL, J. R., RUIZ-OPAZO, N., LEVENSON, R. & NADAL-GINARD, B. (1987) Three differentially expressed Na,K-ATPase alpha subunit isoforms: structural and functional implications. *J Cell Biol*, 105, 1855-65.
- HESS, D. T., MATSUMOTO, A., KIM, S. O., MARSHALL, H. E. & STAMLER, J. S. (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*, 6, 150-66.
- HEVEL, J. M. & MARLETTA, M. A. (1992) Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry*, 31, 7160-5.
- HINGTGEN, S. D., TIAN, X., YANG, J., DUNLAY, S. M., PEEK, A. S., WU, Y., SHARMA, R. V., ENGELHARDT, J. F. & DAVISSON, R. L. (2006) Nox2-containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. *Physiol Genomics*, 26, 180-91.
- HOFFMANN, C., LEITZ, M. R., OBERDORF-MAASS, S., LOHSE, M. J. & KLOTZ, K. N. (2004) Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*, 369, 151-9.
- HOOL LC, GRAY DF, ROBINSON BG & RASMUSSEN HH (1996) Angiotensin-converting enzyme inhibitors regulate the Na(+)-K+ pump via effects on angiotensin metabolism. *Am J Physiol*, 271, C172-C180.
- HOOL, L. C., WHALLEY, D. W., DOOHAN, M. M. & RASMUSSEN, H. H. (1995) Angiotensin-converting enzyme inhibition, intracellular Na+, and Na(+)-K+ pumping in cardiac myocytes. *Am J Physiol*, 268, C366-75.
- HOUSER, S. R. & LAKATTA, E. G. (1999) Function of the cardiac myocyte in the conundrum of end-stage, dilated human heart failure. *Circulation*, 99, 600-4.
- HUANG, B., BLANCO, G., MERCER, R. W., FLEMING, T. & PEPOSE, J. S. (2003) Human corneal endothelial cell expression of Na+,K+-adenosine triphosphatase isoforms. *Arch Ophthalmol*, 121, 840-5.
- HUANG, N. K., LIN, Y. W., HUANG, C. L., MESSING, R. O. & CHERN, Y. (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. *J Biol Chem*, 276, 13838-46.
- IDE, T., TSUTSUI, H., KINUGAWA, S., UTSUMI, H., KANG, D., HATTORI, N., UCHIDA, K., ARIMURA, K., EGASHIRA, K. & TAKESHITA, A. (1999)

- Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res*, 85, 357-63.
- IKEHARA, T., YAMAGUCHI, H., HOSOKAWA, K., TAKAHASHI, A. & MIYAMOTO, H. (1993) Kinetic study on the effects of intracellular K⁺ and Na⁺ on Na⁺, K⁺, Cl⁻ cotransport of HeLa cells by Rb⁺ influx determination. *J Membr Biol*, 132, 115-24.
- IVANOV, A., ZHAO, H. & MODYANOV, N. N. (2000) Packing of the transmembrane helices of Na,K-ATPase: direct contact between beta-subunit and H8 segment of alpha-subunit revealed by oxidative cross-linking. *Biochemistry*, 39, 9778-85.
- JIA, L. G., DONNET, C., BOGAEV, R. C., BLATT, R. J., MCKINNEY, C. E., DAY, K. H., BERR, S. S., JONES, L. R., MOORMAN, J. R., SWEADNER, K. J. & TUCKER, A. L. (2005) Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice. *Am J Physiol Heart Circ Physiol*, 288, H1982-8.
- JOHNSON, F. & GIULIVI, C. (2005) Superoxide dismutases and their impact upon human health. *Mol Aspects Med*, 26, 340-52.
- KANAI, A. J., MESAROS, S., FINKEL, M. S., ODDIS, C. V., BIRDER, L. A. & MALINSKI, T. (1997) Beta-adrenergic regulation of constitutive nitric oxide synthase in cardiac myocytes. *Am J Physiol*, 273, C1371-7.
- KARLISH, S. J., YATES, D. W. & GLYNN, I. M. (1978) Elementary steps of the (Na⁺ + K⁺)-ATPase mechanism, studied with formycin nucleotides. *Biochim Biophys Acta*, 525, 230-51.
- KLEIN, L., O'CONNOR, C. M., GATTIS, W. A., ZAMPINO, M., DE LUCA, L., VITARELLI, A., FEDELE, F. & GHEORGHIADU, M. (2003) Pharmacologic therapy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations. *Am J Cardiol*, 91, 18F-40F.
- KOCKSKAMPER, J., ERLenkAMP, S. & GLITSCH, H. G. (2000) Activation of the cAMP-protein kinase A pathway facilitates Na⁺ translocation by the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol*, 523 Pt 3, 561-74.
- KOCKSKAMPER, J., SENDHOFF, K., ERLenkAMP, S., BORDUSA, F., CEROVSKY, V. & GLITSCH, H. G. (2001) Differences in the protein-kinase-A-dependent regulation of CFTR Cl⁻ channels and Na⁺-K⁺ pumps in guinea-pig ventricular myocytes. *Pflugers Arch*, 441, 807-15.
- KOJIMA, H., NAKATSUBO, N., KIKUCHI, K., KAWAHARA, S., KIRINO, Y., NAGOSHI, H., HIRATA, Y. & NAGANO, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem*, 70, 2446-53.
- KONG, B. Y. & CLARKE, R. J. (2004) Identification of potential regulatory sites of the Na⁺,K⁺-ATPase by kinetic analysis. *Biochemistry*, 43, 2241-50.
- KOU, R. & MICHEL, T. (2007) Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*, 282, 32719-29.
- KURZ, S. & HARRISON, D. G. (1997) Insulin and the arginine paradox. *J Clin Invest*, 99, 369-70.

- LAMBETH, J. D., KAWAHARA, T. & DIEBOLD, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med*, 43, 319-31.
- LASS, A., SUESSENBACHER, A., WOLKART, G., MAYER, B. & BRUNNER, F. (2002) Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol*, 61, 1081-8.
- LASTRA, G. & MANRIQUE, C. (2007) The expanding role of oxidative stress, renin angiotensin system, and beta-cell dysfunction in the cardiometabolic syndrome and Type 2 diabetes mellitus. *Antioxid Redox Signal*, 9, 943-54.
- LAUGER, P. (1991) Kinetic basis of voltage dependence of the Na,K-pump. *Soc Gen Physiol Ser*, 46, 303-15.
- LEMAY, J., HOU, Y. & DEBLOIS, D. (2000) Evidence that nitric oxide regulates AT1-receptor agonist and antagonist efficacy in rat injured carotid artery. *J Cardiovasc Pharmacol*, 35, 693-9.
- LERMAN, L. O. & LERMAN, A. (2007) All oxidase roads lead to angiotensin, too. *Arterioscler Thromb Vasc Biol*, 27, 703-4.
- LEUNG, P. S. (2004) The peptide hormone angiotensin II: its new functions in tissues and organs. *Curr Protein Pept Sci*, 5, 267-73.
- LI, J. M. & SHAH, A. M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol*, 287, R1014-30.
- LIU, C., FIGTREE, G. A., HAMILTON, E. J., GARCIA, A., WHITE, C. N., CHIA, K. K. M., CORNELIUS, F. & RAMUSSEN, H. H. (2008) Oxidative signalling inhibits the cardiac Na⁺-K⁺ pump via glutathionylation. *submitted*.
- LIU, L. & ASKARI, A. (2006) beta-Subunit of cardiac Na⁺-K⁺-ATPase dictates the concentration of the functional enzyme in caveolae. *Am J Physiol Cell Physiol*, 291, C569-78.
- LIU, L., MOHAMMADI, K., AYNASHAR, B., WANG, H., LI, D., LIU, J., IVANOV, A. V., XIE, Z. & ASKARI, A. (2003) Role of caveolae in signal-transducing function of cardiac Na⁺/K⁺-ATPase. *Am J Physiol Cell Physiol*, 284, C1550-60.
- LUPFERT, C., GRELL, E., PINTSCHOVIVUS, V., APELL, H. J., CORNELIUS, F. & CLARKE, R. J. (2001) Rate limitation of the Na⁺,K⁺-ATPase pump cycle. *Biophys J*, 81, 2069-81.
- LUTSENKO, S. & KAPLAN, J. H. (1995) Organization of P-type ATPases: significance of structural diversity. *Biochemistry*, 34, 15607-13.
- MAACK, C., KARTES, T., KILTER, H., SCHAFERS, H. J., NICKENIG, G., BOHM, M. & LAUFS, U. (2003) Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation*, 108, 1567-74.
- MALIK, N., CANFIELD, V. A., BECKERS, M. C., GROS, P. & LEVENSON, R. (1996) Identification of the mammalian Na,K-ATPase 3 subunit. *J Biol Chem*, 271, 22754-8.
- MANN, G. E., YUDILEVICH, D. L. & SOBREVIA, L. (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev*, 83, 183-252.

- MARGOLIS, A. S., PORASUPHATANA, S. & ROSEN, G. M. (2000) Role of paraquat in the uncoupling of nitric oxide synthase. *Biochim Biophys Acta*, 1524, 253-7.
- MARTIN-VASALLO, P., DACKOWSKI, W., EMANUEL, J. R. & LEVENSON, R. (1989) Identification of a putative isoform of the Na,K-ATPase beta subunit. Primary structure and tissue-specific expression. *J Biol Chem*, 264, 4613-8.
- MARTINEZ-RUIZ, A. & LAMAS, S. (2007) Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences. *Cardiovasc Res*, 75, 220-8.
- MASSION PB, F. O., DESSY C, BALLIGAND JL (2003) Nitric oxide and cardiac function: ten years after, and continuing. *Circ Res*. . , 93, 388-98.
- MATHERS, J., FRASER, J. A., MCMAHON, M., SAUNDERS, R. D., HAYES, J. D. & MCLELLAN, L. I. (2004) Antioxidant and cytoprotective responses to redox stress. *Biochem Soc Symp*, 157-76.
- MATSUDA, H. & IYANAGI, T. (1999) Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. *Biochim Biophys Acta*, 1473, 345-55.
- MAYER, B., JOHN, M., HEINZEL, B., WERNER, E. R., WACHTER, H., SCHULTZ, G. & BOHME, E. (1991) Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett*, 288, 187-91.
- MERCER, R. W., BIEMESDERFER, D., BLISS, D. P., JR., COLLINS, J. H. & FORBUSH, B., 3RD (1993) Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol*, 121, 579-86.
- MIHAILIDOU, A. S., BUNDGAARD, H., MARDINI, M., HANSEN, P. S., KJELDSSEN, K. & RASMUSSEN, H. H. (2000) Hyperaldosteronemia in rabbits inhibits the cardiac sarcolemmal Na(+)-K(+) pump. *Circ Res*, 86, 37-42.
- MOHAN, P., BRUTSAERT, D. L., PAULUS, W. J. & SYS, S. U. (1996) Myocardial contractile response to nitric oxide and cGMP. *Circulation*, 93, 1223-9.
- MORGAN T (2003) Renin, angiotensin, sodium and organ damage. *Hypertens Res*, 26, 349-54.
- MORTH, J. P., PEDERSEN, B. P., TOUSTRUP-JENSEN, M. S., SORENSEN, T. L., PETERSEN, J., ANDERSEN, J. P., VILSEN, B. & NISSEN, P. (2007) Crystal structure of the sodium-potassium pump. *Nature*, 450, 1043-9.
- MUDD, J. O. & KASS, D. A. (2008) Tackling heart failure in the twenty-first century. *Nature*, 451, 919-28.
- MUNSON, K., LAMBRECHT, N., SHIN, J. M. & SACHS, G. (2000) Analysis of the membrane domain of the gastric H(+)/K(+)-ATPase. *J Exp Biol*, 203, 161-70.
- MURDOCH, C. E., GRIEVE, D. J., CAVE, A. C., LOOI, Y. H. & SHAH, A. M. (2006) NADPH oxidase and heart failure. *Curr Opin Pharmacol*, 6, 148-53.

- MURIEL, P., CASTANEDA, G., ORTEGA, M. & NOEL, F. (2003) Insights into the mechanism of erythrocyte Na⁺/K⁺-ATPase inhibition by nitric oxide and peroxyxynitrite anion. *J Appl Toxicol*, 23, 275-8.
- MURIEL P, S. G. (2000) Nitric oxide and peroxyxynitrite anion modulate liver plasma membrane fluidity and Na⁽⁺⁾/K⁽⁺⁾-ATPase activity. *Nitric Oxide*.2, 4, 333- 342.
- NAKAO, M. & GADSBY, D. C. (1989) [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J Gen Physiol*, 94, 539-65.
- NEUBAUER, S. (2007) The failing heart--an engine out of fuel. *N Engl J Med*, 356, 1140-51.
- OHARA, Y., PETERSON, T. E. & HARRISON, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*, 91, 2546-51.
- PACKER, M., CARVER, J. R., RODEHEFFER, R. J., IVANHOE, R. J., DIBIANCO, R., ZELDIS, S. M., HENDRIX, G. H., BOMMER, W. J., ELKAYAM, U., KUKIN, M. L. & ET AL. (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. *N Engl J Med*, 325, 1468-75.
- PARK, J. W., HOYAL, C. R., BENNA, J. E. & BABIOR, B. M. (1997) Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(PHOX) during oxidase activation. *J Biol Chem*, 272, 11035-43.
- PELUFFO, R. D., ARGUELLO, J. M. & BERLIN, J. R. (2000) The role of Na,K-ATPase alpha subunit serine 775 and glutamate 779 in determining the extracellular K⁺ and membrane potential-dependent properties of the Na,K-pump. *J Gen Physiol*, 116, 47-59.
- PELUFFO, R. D., HARA, Y. & BERLIN, J. R. (2004) Quaternary organic amines inhibit Na,K pump current in a voltage-dependent manner: direct evidence of an extracellular access channel in the Na,K-ATPase. *J Gen Physiol*, 123, 249-63.
- PETRUSHANKO, I., BOGDANOV, N., BULYGINA, E., GRENACHER, B., LEINSOO, T., BOLDYREV, A., GASSMANN, M. & BOGDANOVA, A. (2006) Na-K-ATPase in rat cerebellar granule cells is redox sensitive. *Am J Physiol Regul Integr Comp Physiol*, 290, R916-25.
- PIESKE B, MAIER LS, PIACENTINO V 3RD, WEISSER J, HASENFUSS G & HOUSER S (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*., 106, 447-453.
- PIESKE, B. & HOUSER, S. R. (2003) [Na⁺]_i handling in the failing human heart. *Cardiovasc Res*, 57, 874-86.
- PIESKE, B., HOUSER, S. R., HASENFUSS, G. & BERS, D. M. (2003) Sodium and the heart: a hidden key factor in cardiac regulation. *Cardiovasc Res*, 57, 871-2.
- PIESKE, B., MAIER, L. S., PIACENTINO, V., 3RD, WEISSER, J., HASENFUSS, G. & HOUSER, S. (2002) Rate dependence of [Na⁺]_i and contractility in nonfailing and failing human myocardium. *Circulation*, 106, 447-53.
- PLUMB, R. D., EL-SHERBEENY, N. A., DIXON, L. J., HUGHES, S. M., DEVINE, A. B., LEAHEY, W. J. & MCVEIGH, G. E. (2005) NAD(P)H-

- dependent superoxide production in platelets: the role of angiotensin II and protein kinase C. *Clin Biochem*, 38, 607-13.
- POGWIZD SM, SIPIDO KR, VERDONCK F & BERS DM (2003) Intracellular Na in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. *Cardiovasc Res*, 57, 887-896.
- RAJU, S. V., BAROUCH, L. A. & HARE, J. M. (2005) Nitric oxide and oxidative stress in cardiovascular aging. *Sci Aging Knowledge Environ*, 2005, re4.
- RAKOWSKI, R. F., BEZANILLA, F., DE WEER, P., GADSBY, D. C., HOLMGREN, M. & WAGG, J. (1997a) Charge translocation by the Na/K pump. *Ann N Y Acad Sci*, 834, 231-43.
- RAKOWSKI, R. F., GADSBY, D. C. & DE WEER, P. (1997b) Voltage dependence of the Na/K pump. *J Membr Biol*, 155, 105-12.
- RAO, G. N., SARDET, C., POUYSSEGUR, J. & BERK, B. C. (1990) Differential regulation of Na⁺/H⁺ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J Biol Chem*, 265, 19393-6.
- REY, F. E., CIFUENTES, M. E., KIARASH, A., QUINN, M. T. & PAGANO, P. J. (2001) Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O₂(⁻) and systolic blood pressure in mice. *Circ Res*, 89, 408-14.
- ROMERO-ALVIRA, D. & ROCHE, E. (1996) High blood pressure, oxygen radicals and antioxidants: etiological relationships. *Med Hypotheses*, 46, 414-20.
- RUSSELL, J. M. (2000) Sodium-potassium-chloride cotransport. *Physiol Rev*, 80, 211-76.
- RYBIN, V. O., XU, X. & STEINBERG, S. F. (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae : stimulation of local protein phosphorylation. *Circ Res*, 84, 980-8.
- SAGAR, A. & RAKOWSKI, R. F. (1994) Access channel model for the voltage dependence of the forward-running Na⁺/K⁺ pump. *J Gen Physiol*, 103, 869-93.
- SATO, T., KAMATA, Y., IRIFUNE, M. & NISHIKAWA, T. (1997) Inhibitory effect of several nitric oxide-generating compounds on purified Na⁺,K⁽⁺⁾-ATPase activity from porcine cerebral cortex. *J Neurochem*, 68, 1312-8.
- SEDDON, M., LOOI, Y. H. & SHAH, A. M. (2007) Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart*, 93, 903-7.
- SHAH, A. M. & MACCARTHY, P. A. (2000) Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther*, 86, 49-86.
- SHAINSKAYA, A., SCHNEEBERGER, A., APELL, H. J. & KARLISH, S. J. (2000) Entrance port for Na⁽⁺⁾ and K⁽⁺⁾ ions on Na⁽⁺⁾,K⁽⁺⁾-ATPase in the cytoplasmic loop between trans-membrane segments M6 and M7 of the alpha subunit. Proximity Of the cytoplasmic segment of the beta subunit. *J Biol Chem*, 275, 2019-28.
- SHAMRAJ, O. I. & LINGREL, J. B. (1994) A putative fourth Na⁺,K⁽⁺⁾-ATPase alpha-subunit gene is expressed in testis. *Proc Natl Acad Sci U S A*, 91, 12952-6.
- SHIMON, M. B., GOLDSHLEGER, R. & KARLISH, S. J. (1998) Specific Cu²⁺-catalyzed oxidative cleavage of Na,K-ATPase at the extracellular surface. *J Biol Chem*, 273, 34190-5.

- SHINODA, T., OGAWA, H., CORNELIUS, F. & TOYOSHIMA, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature*, 459, 446-50.
- SILVERMAN, B. Z., FULLER, W., EATON, P., DENG, J., MOORMAN, J. R., CHEUNG, J. Y., JAMES, A. F. & SHATTOCK, M. J. (2005) Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. *Cardiovasc Res*, 65, 93-103.
- SIRAGY, H. M. & CAREY, R. M. (1997) The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*, 100, 264-9.
- SKOU, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta*, 23, 394-401.
- SKOU, J. C. (1998) Nobel Lecture. The identification of the sodium pump. *Biosci Rep*, 18, 155-69.
- SOMERS, M. J., MAVROMATIS, K., GALIS, Z. S. & HARRISON, D. G. (2000) Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation*, 101, 1722-8.
- STADTMAN, E. R. & LEVINE, R. L. (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, 25, 207-18.
- STANTON, B. A. & KAISLING, B. (1989) Regulation of renal ion transport and cell growth by sodium. *Am J Physiol*, 257, F1-10.
- STOLK, J., HILTERMANN, T. J., DIJKMAN, J. H. & VERHOEVEN, A. J. (1994) Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*, 11, 95-102.
- STUEHR, D. J., CHO, H. J., KWON, N. S., WEISE, M. F. & NATHAN, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, 88, 7773-7.
- SWEADNER, K. J. (2005) Phospholemman: a new force in cardiac contractility. *Circ Res*, 97, 510-1.
- SWEADNER, K. J., ARYSTARKHOVA, E., DONNET, C. & WETZEL, R. K. (2003) FXYD proteins as regulators of the Na,K-ATPase in the kidney. *Ann N Y Acad Sci*, 986, 382-7.
- SWEADNER, K. J. & FESCHENKO, M. S. (2001) Predicted location and limited accessibility of protein kinase A phosphorylation site on Na-K-ATPase. *Am J Physiol Cell Physiol*, 280, C1017-26.
- SWEADNER, K. J. & RAEL, E. (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics*, 68, 41-56.
- SWEDBERG, K., ENEROTH, P., KJEKSHUS, J. & WILHELMSSEN, L. (1990) Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group. *Circulation*, 82, 1730-6.
- SYDOW, K. & MUNZEL, T. (2003) ADMA and oxidative stress. *Atheroscler Suppl*, 4, 41-51.

- SZABO, C. (2003) Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett*, 140-141, 105-12.
- TAMURA, N., KONISHI, S., IWAKI, S., KIMURA-SOMEYA, T., NADA, S. & YAMAGUCHI, A. (2001) Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J Biol Chem*, 276, 20330-9.
- TAUB, M., BORSICK, M., GEISEL, J., MATLHAGELA, K., RAJKHOWA, T. & ALLEN, C. (2004) Regulation of the Na,K-ATPase in MDCK cells by prostaglandin E1: a role for calcium as well as cAMP. *Exp Cell Res*, 299, 1-14.
- TEN HOVE, M., CHAN, S., LYGATE, C., MONFARED, M., BOEHM, E., HULBERT, K., WATKINS, H., CLARKE, K. & NEUBAUER, S. (2005) Mechanisms of creatine depletion in chronically failing rat heart. *J Mol Cell Cardiol*, 38, 309-13.
- THERIEN, A. G. & BLOSTEIN, R. (1999) K(+)/Na(+) antagonism at cytoplasmic sites of Na(+)-K(+)-ATPase: a tissue-specific mechanism of sodium pump regulation. *Am J Physiol*, 277, C891-8.
- THERIEN, A. G. & BLOSTEIN, R. (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*, 279, C541-66.
- TODA, N., AYAJIKI, K. & OKAMURA, T. (2007) Interaction of endothelial nitric oxide and angiotensin in the circulation. *Pharmacol Rev*, 59, 54-87.
- TOURNEUR, Y., MITRA, R., MORAD, M. & ROUGIER, O. (1987) Activation properties of the inward-rectifying potassium channel on mammalian heart cells. *J Membr Biol*, 97, 127-35.
- TSUTSUI, H. (2004) Novel Pathophysiological Insight and Treatment Strategies for Heart Failure. *Circ J*, 68, 1095-1103.
- TURKO, I. V. & MURAD, F. (2002) Protein nitration in cardiovascular diseases. *Pharmacol Rev*, 54, 619-34.
- VARELA, M., HERRERA, M. & GARVIN, J. L. (2004) Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on O₂⁻ and is diminished by a high-salt diet. *Am J Physiol Renal Physiol*, 287, F224-30.
- VASQUEZ-VIVAR, J., MARTASEK, P., WHITSETT, J., JOSEPH, J. & KALYANARAMAN, B. (2002) The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J*, 362, 733-9.
- VAUGHAN-JONES, R. D., EISNER, D. A. & LEDERER, W. J. (1985) The effects of intracellular Na on contraction and intracellular pH in mammalian cardiac muscle. *Adv Myocardiol*, 5, 313-30.
- VIGNERY, A., WANG, F., QIAN, H. Y., BENZ, E. J., JR. & GILMORE-HEBERT, M. (1991) Detection of the Na(+)-K(+)-ATPase alpha 3-isoform in multinucleated macrophages. *Am J Physiol*, 260, F704-9.
- VILA-PETROFF, M. G., YOUNES, A., EGAN, J., LAKATTA, E. G. & SOLLOTT, S. J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res*, 84, 1020-31.
- VUKOSAVLJEVIC, N., JARON, D., BARBEE, K. A. & BUERK, D. G. (2006) Quantifying the L-arginine paradox in vivo. *Microvasc Res*, 71, 48-54.
- WANG, F., CHEN, C. C., MAO, B. Y., MAO, Q., ZHANG, Y. K., LEI, D. & XU, R. X. (2004) [Effect of L-arginine on the changes of cerebral microcirculation

- following craniocerebral missile wound at early stage in cats]. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 35, 402-5.
- WANG, Y., GAO, J., MATHIAS, R. T., COHEN, I. S., SUN, X. & BALDO, G. J. (1998) alpha-Adrenergic effects on Na⁺-K⁺ pump current in guinea-pig ventricular myocytes. *J Physiol*, 509 (Pt 1), 117-28.
- WARNHOLTZ, A., NICKENIG, G., SCHULZ, E., MACHARZINA, R., BRASEN, J. H., SKATCHKOV, M., HEITZER, T., STASCH, J. P., GRIENGLING, K. K., HARRISON, D. G., BOHM, M., MEINERTZ, T. & MUNZEL, T. (1999) Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*, 99, 2027-33.
- WENZEL-SEIFERT, K., LIU, H. Y. & SEIFERT, R. (2002) Similarities and differences in the coupling of human beta1- and beta2-adrenoceptors to Gs(alpha) splice variants. *Biochem Pharmacol*, 64, 9-20.
- WHITE, C. N., FIGTREE, G. A., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K. & RASMUSSEN, H. H. (2009) Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol*, 296, C693-700.
- WHITE, C. N., HAMILTON, E. J., GARCIA, A., WANG, D., CHIA, K. K. M., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) Opposing effects of coupled and uncoupled NOS activity on the Na⁺-K⁺ pump in cardiac myocytes. *Am J Physiol Cell Physiol*, 294, C572-8.
- WHITE, C. N., LIU, C. C., GARCIA, A., HAMILTON, E. J., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2010) Activation of cAMP-dependent signaling induces oxidative modification of the cardiac Na⁺-K⁺ pump and inhibits its activity. *J Biol Chem*, 285, 13712-20.
- WILLIAM, M., HAMILTON, E. J., GARCIA, A., BUNDGAARD, H., CHIA, K. K., FIGTREE, G. A. & RASMUSSEN, H. H. (2008) Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation. *Am J Physiol Cell Physiol*, 294, C1067-73.
- WILLIAM, M., VIEN, J., HAMILTON, E., GARCIA, A., BUNDGAARD, H., CLARKE, R. J. & RASMUSSEN, H. H. (2005) The nitric oxide donor, sodium nitroprusside, stimulates the Na⁺-K⁺ pump in isolated rabbit cardiac myocytes. *J Physiol*, 565, 815-25.
- WILLIAMS, H. C. & GRIENGLING, K. K. (2007) NADPH oxidase inhibitors: new antihypertensive agents? *J Cardiovasc Pharmacol*, 50, 9-16.
- WINTERBOURN, C. C. & HAMPTON, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med*, 45, 549-61.
- XIE Z, KOMETIANI P, LIU J, LI J, SHAPIRO JI & ASKARI A (1999) Intracellular reactive oxygen species mediate the linkage of Na⁺/K⁺-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem*, 274, 19323-19328.
- YANG, B. & RIZZO, V. (2007) TNF- α potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*, 292, H954-962.
- YAO, L., FAN, P., JIANG, Z., GORDON, A., MOCHLY-ROSEN, D. & DIAMOND, I. (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-

- dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol*, 73, 1105-12.
- ZAUGG, M., LUCCHINETTI, E., UECKER, M., PASCH, T. & SCHAUB, M. C. (2003) Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth*, 91, 551-65.
- ZHANG, C., IMAM, S. Z., ALI, S. F. & MAYEUX, P. R. (2002) Peroxynitrite and the regulation of Na(+),K(+)-ATPase activity by angiotensin II in the rat proximal tubule. *Nitric Oxide*, 7, 30-5.
- ZHAO, X., XU, B., BHATTACHARJEE, A., OLDFIELD, C. M., WIENTJES, F. B., FELDMAN, G. M. & CATHCART, M. K. (2005) Protein kinase Cdelta regulates p67phox phosphorylation in human monocytes. *J Leukoc Biol*, 77, 414-20.