VOLUME-SENSITIVE K^+ CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES

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SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
AT
DALHOUSIE UNIVERSITY
HALIFAX, NOVA SCOTIA
SEPTEMBER 2005

Your file Votre référence
ISBN: 978-0-494-16701-4
Our file Notre référence
ISBN: 978-0-494-16701-4

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to my wife

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Abstract

The slow component of cardiac delayed-rectifier potassium current (I_{Ks}) is active under physiological conditions in guinea pig ventricular cardiac myocytes and plays a role in myocyte action potential plateau termination and membrane repolarization. I_{Ks} is stimulated by application of hyposmotic external solution and inhibited by application of hyperosmotic solution. The mechanism responsible for this modulation remains largely unknown. The goal of the present study was to investigate the role of tyrosine phosphorylation/dephosphorylation in the stimulation of I_{Ks} by hyposmotic solution and to obtain pertinent information on regulation of I_{Ks} under isosmotic conditions.

Application of hyposmotic (0.75T) solution increased the amplitude of I_{Ks} by 73%, with little effect on current kinetics and the voltage-dependence of activation. Protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol-3-kinase (PI3-K), MAP kinase (ERK1/2), G-proteins, and the cytoskeleton appear not to be involved in the stimulation of I_{Ks} by hyposmotic solution. On the other hand, broadspectrum protein tyrosine kinase (PTK) inhibitors genistein, tyrphostin A23, and tyrphostin A25 strongly decreased the amplitude of I_{Ks} under isosmotic conditions with EC_{50} values of 64, 4.1, and 12.3 μM, respectively. These inhibitors also blunted stimulation of the current by hyposmotic solution. Src PTK inhibitor PP2 decreased I_{Ks} under isosmotic conditions and attenuated hyposmotic stimulation of I_{Ks}, whereas epidermal growth factor receptor (EGFR) inhibitor tyrphostin AG1478 had neither of these effects. PTK-inactive analogues of genistein (genistin and daidzein), tyrphostins (A1 and A63), and PP2 (PP3) had little or no inhibitory influence on I_{Ks}.

The phosphotyrosyl phosphatase (PTP) inhibitors orthovanadate (1 mM) and DMHV (100 μM) had little effect on I_{Ks} under control conditions. However, they rapidly reversed the inhibitory effects of genistein, A23, and A25 on I_{Ks}. These results suggest that I_{Ks} in guinea pig ventricular myocytes is strongly modulated by PTK/PTP under isosmotic conditions, and that the stimulation of I_{Ks} by hyposmotic solution depends on Src-type PTK activity. Stimulation of I_{Ks} under hyposmotic conditions decreases action potential duration, a potential antiarrhythmogenic effect.

List of Abbreviations and Symbols Used

A ampere

AC adenylate cyclase

ACh acetylcholine

AKAP A-kinase anchoring protein

APD action potential duration

ATP adenosine triphosphate

BHK baby hamster kidney cells

Bis bisindolylmaleimide I

cAMP adenosine 3',5'-cyclic monophosphate

cDNA complementary DNA

CFTR cystic fibrosis transmembrane conductance regulator

cGMP guanosine 3',5'-cyclic monophosphate

CHO Chinese hamster ovary cells

C_m membrane capacitance

COS African green monkey kidney fibroblast-like cells

CytD cytochalasin D

DDZ daidzein

DIDS 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid

DI diastolic interval

DMHV bis-(N,N-dimethylhydroxamido) hydroxooxovanadate

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

EC_{50} concentration of the drug that produces 50% of the maximal effect

ECM extracellular matrix

EGFR epidermal growth factor receptor

ECG electrocardiogram

EGTA ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ERK extracellular receptor kinase

FAK focal adhesion kinase

FSK forskolin

g gram

GDP guanosine-5'-diphosphate

GDPβS guanosine-5'-0-(2-thiodiphosphate)

GFP green fluorescence protein

GPCR G-protein coupled receptor

GST genistein

GTN genistin

GTP guanosine-5'-triphosphate

H7 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine

H8 N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide

H89 N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide

HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HOG high osmolarity glycerol

Hz herz

I current

I_{Ba} Ba^{2+} current through L-type Ca^{2+} channels

$I_{Ca,\,L}$ L-type calcium current

$I_{Cl,\,vol}$ volume-sensitive chloride current

I_{K1} inwardly-rectifying potassium current

$I_{K,\,ATP}$ ATP-dependent potassium current

I_{Kr} rapidly-activating delayed rectifier potassium current

I_{Ks} slowly-activating delayed rectifier potassium current

I_{Na} sodium current

IP_3 inositol 1,4,5-triphosphate

ISO isoproterenol

I–V current–voltage

Kir inwardly-rectifying potassium channel

Kv voltage-dependent potassium channel

LQT long QT syndrome

M moles per liter

MAPK mitogen-activated protein kinase

MDCK Madin-Darby canine kidney cells

min minute

mRNA messenger RNA

n number of experiments

n_H Hill coefficient

NMDA N-methyl-D-aspartate

OA okadeic acid

p probability

P2Y purinergic receptors

PDE3 phosphodiesterase 3

PDGFR platelet-derived growth factor receptor

pH negative logarithm of the hydrogen ion concentration

PI3-K phosphatidylinositol-3-kinase

PI4-K phosphatidylinositol-4-kinase

PI phosphatidylinositol

PIP_2 phosphatidylinositol 4,5-bisphosphate

PKA protein kinase A

PKC protein kinase C

PKG protein kinase G

PLC phospholipase C

PMA phorbol-12-myristate-13-acetate

PP2 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-D]pyrimidine

PP3 4-amino-7-phenylpyrazol[3,4-D]pyrimidine

PTK protein tyrosine kinase

PTP phosphotyrosine phosphatase

PTX pertussis toxin

Q_{10} temperature coefficient

s second

S slope factor of Boltzmann function

RNA ribonucleic acid

RTK receptor tyrosine kinase

RVD regulatory volume decrease

RVI regulatory volume increase

SUR sulfonylurea receptor

TEA tetraethylammonium

TMD trans-membrane domain

τ time constant

V volt

Van sodium orthovanadate

$V_{0.5}$ half-maximal potential

[X] concentration of ion X

$\approx$ approximately

°C degree Celsius

Ω ohm

% per cent

Acknowledgements

I would like to sincerely thank my supervisor, Dr. T.F. McDonald, for his guidance, support, and friendship throughout the course of my work at Dalhousie.

I would like to extend thanks to the members of my supervisory committee, Drs. Melanie Kelly, Paul Linsdell, and Alfredo Villarroel, and my examining committee (Drs. Valérie Chappe, Dieter Pelzer, and Alvin Shrier) for their time and guidance.

I would like to thank Mrs. Gina Dickie, Mr. Brian Hoyt, Mr. Mark Richard and Ms. Alice Smith for their excellent assistance and technical support.

I am very grateful to the fellow lab members Lesja, Steven, Oksana, Pavel, and Chi for their help and friendship. I would also like to thank members of Dr. Paul Linsdell's lab, Ms. Susan Burbridge and Dr. Jennifer Killey, for their assistance and guidance.

I want to thank my loving wife Olja and family back in Kiev for their patience and encouragement during these long years.

1

Introduction

Potassium channels comprise an extremely diverse group of membrane-spanning proteins expressed in viruses (Gazzarrini *et al.*, 2003; Kang *et al.*, 2004), prokaryotes (Milkman, 1994), and eukaryotes, including plants (Zimmermann and Sentenac, 1999) and animals. The functions that these channels perform are also very diverse: they play a role in membrane potential maintenance, excitability, ion concentration homeostasis, membrane ion transport, and cell volume regulation.

More than 200 K^+ channel-related genes have been identified to date. The human genome contain more than 110 K^+ channel-related genes (Entrez Genome metrics). Some of the genes encode pore-forming α-subunits, each of which contains a selectivity filter that allows K^+ ions to cross the membrane at a high rate, and a gating machinery that controls the opening and closing of channels in response to changes in voltage (Sigworth, 1994; Bezanilla, 2000) or to binding of a chemical ligand (Wickman and Clapham, 1995), while others encode auxiliary β-subunits that modulate channel properties.

1.1 Molecular structure of potassium channels

In recent years, a great deal has been discovered about the structure and function of K^+ channels. Structurally, all K^+ channels repeat the same basic configuration: in the simplest case, four homogeneous subunits form the channel pore (Doyle *et al.*, 1998; Zhou *et al.*, 2001). The pore is the passage through which the K^+ ions cross the cell membrane. K^+ channels are extremely selective, i.e., they favor the passage of K^+ ions over other cytoplasmic and extracellular cations. At the same time, the channels allow K^+ permeation at a throughput rate (10^6 to 10^8 K^+ ions/s) that is close to the theoretical diffusion limit.

K^+ channels can be subdivided into the two large groups: voltage-gated K^+ channels (Kv), and inward-rectifier K^+ channels (Kir). Based on the topology of the transmembrane domains of their α-subunits, four families of K^+ channels have been described.

The largest family of Kv channels is termed 6TMD-1P; it is comprised of channels whose α-subunits have 6 trans-membrane domains (TMDs) (S1–S6) and one pore-forming (P) loop. The pore of the channels is formed by parts of TMDs S5 and S6 and the P loop. The P-loop forms the outer part of the channel pore and contains the K^+ channel selectivity signature T/SxxTxGYG (Heginbotham *et al.*, 1994). The 4th TMD contains positively-charged amino acid residues and plays a role in channel gating by voltage. The C-terminus of the protein has a highly-conserved motif (A-domain) that has sites that can be phosphorylated by protein kinases and bound by Ca^{2+}. Members of the 6TMD-1P family include classical *Shaker* channels (Salkoff, 1983), as well as erg (Sanguinetti *et al.*, 1995) and KCNQ channels that play a role in cardiac repolarization.

Large conductance Ca^{2+}-activated K^+ channels (maxi-K^+) belong to the 7TMD-1P family. Some data indicate the presence of the 7th TMD at the N-terminus of the channel, called S0 (Meera *et al.*, 1997).

2TMD-1P channels belong to the Kir group. These channels are of much simpler organization than the voltage-gated K^+ channels, and contain just two TMDs linked by the P-loop in each subunit. Similar to Kv channels, four subunits bind together to form a functional Kir channel. In some cases, members of the 2TMD-1P family coassemble with large proteins, such as sulfonylurea receptors (SUR) (Repunte *et al.*, 1999) and CFTR (Ruknudin *et al.*, 1998), to form ATP-sensitive channels. Members of this family include the channels (Kir 2.x, or KCNJ) that carry inward-rectifier K^+ current (I_{K1}) in the heart (Raab-Graham *et al.*, 1994), ATP-sensitive channels (Kir 6.1 or Kir 6.2 + SUR) (Cui *et al.*, 2001), and muscarine-gated K^+ channels (Tucker *et al.*, 1996).

Channels that belong to the 4TMD-2P family contain 2 P-loops per subunit, suggesting that either two (instead of four) subunits form a functional channel, or that the channel has two pores. The channels that belong to this family were cloned from both *Drosophila* (Goldstein *et al.*, 1996) and mammalian genome (Fink *et al.*, 1996; Maingret *et al.*, 2000). The channels exhibit no voltage dependence, and carry a potassium "leak" current. Members of this this family include TWIK, TASK, and TREK. These channels are modulated by a variety of factors including cell pH, arachidonic acid, mechanical stress, anaesthetics, and temperature (Duprat *et al.*, 1997; Fink *et al.*, 1998; Maingret *et al.*, 1999).

1.2 KCNQ potassium channels

1.2.1 Molecular structure

KCNQ channels belong to the 6TMD-1P family of potassium channels, and the amino acid sequence can be used to further differentiate the channels.

The first cloning attempts revealed a gene KvLQT1 (now KCNQ1) on human chromosome 11 (Wang *et al.*, 1996). The full-length human cDNA clone was obtained by Sanguinetti *et al.* (1996), and the length of the protein product was predicted to be 581 amino acids. Using Northern blot, an mRNA for KCNQ1 was found in human pancreas, heart, kidney, lung, and placenta (Sanguinetti *et al.*, 1996). At the same time, Barhanin *et al.* (1996) cloned a full-length cDNA from a mouse heart library. They found that it encodes a 604 amino acid protein with 90.5% similarity to human KCNQ1. Hydrophobicity analysis predicted a 6 TMD structure with a configuration typical of voltage-gated K^+ channels. The length of human KCNQ1 was eventually estimated to be 676 amino acids by Yang *et al.* (1997), and Neyroud *et al.* (1999) found that there are 19 exons in the gene located on chromosome site 11p15.5.

The first transfections of KCNQ1 cDNA into Chinese hamster ovary (CHO) (Sanguinetti *et al.*, 1996) and African green monkey kidney fibroblast-like (COS-7) (Barhanin *et al.*, 1996) cells confirmed that the protein forms functional potassium channels with properties not seen before in K^+ channels. The coexpression of KCNQ1 with auxiliary β-subunit (KCNE1) changed the biophysical properties of the current to those closely resembling cardiac I_{Ks} (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996).

Table 1: Nomenclature and distribution of KCNQ channels.

Gene	Chromosome	Expression	References
KCNQ1	11p15.5	Heart, cochlea, placenta, lung, intestine	(Sanguinetti *et al.*, 1996; Wang *et al.*, 1996)
KCNQ2	20q13.3	Brain	(Biervert *et al.*, 1998; Singh *et al.*, 1998)
KCNQ3	8q23	Brain	(Charlier *et al.*, 1998; Yang *et al.*, 1998)
KCNQ4	1p34	Outer hair cells, central auditory pathway	(Kubisch *et al.*, 1999; Kharkovets *et al.*, 2000)
KCNQ5	6q14	Brain, skeletal muscle	(Lerche *et al.*, 2000; Schroeder *et al.*, 2000)

Homomeric channels

KCNQ1 currents are slowly-activating and have steady-state activation relationships with half-activation voltage ($V_{0.5}$) between -10 and -20 mV and a slope factor of $+12$ mV (Sanguinetti *et al.*, 1996). When KCNQ1 channels are expressed in mammalian cells, the current reaches a steady state level of activation within 1 s (Sanguinetti *et al.*, 1996; Wang *et al.*, 2000; Yang *et al.*, 1997). The deactivation tails exhibit a "hook" behaviour, providing evidence of a fast inactivation masked by a slower deactivation process (Tristani-Firouzi and Sanguinetti, 1998). The nomenclature and information on distribution of homomeric KCNQ channels is provided in Table 1.

The biophysical characteristics of KCNQ2 are quite variable, with $V_{0.5}$ values between -14 and -38 mV and slopes between $+7$ and $+14$ mV. Similar to KCNQ1, KCNQ2 currents activate slowly on depolarization (Schroeder *et al.*, 1998; Wang *et al.*, 1998).

KCNQ3 current has a $V_{0.5}$ of -37 mV and a slope of $+5.5$ mV (Yang *et al.*, 1998; Selyanko *et al.*, 2000). The current undergoes inward rectification at potentials >0

mV, and a crossover pattern of activation is evident with increasing amplitude of the voltage step.

KCNQ4 expression is limited to the inner ear and certain parts of the central auditory tract (Kubisch *et al.*, 1999; Kharkovets *et al.*, 2000). KCNQ4 expressed in CHO cells has a $V_{0.5}$ of -19 mV and a slope of $+10$ mV, and does not exhibit a pronounced inactivation (Selyanko *et al.*, 2000). Slower activation is reported for the channels when they are expressed in *Xenopus* oocytes (Kubisch *et al.*, 1999; Schroeder *et al.*, 2000). Similar to other KCNQ channels, the current is inhibited when coexpressed with M1 muscarinic receptors (Selyanko *et al.*, 2000).

KCNQ5 is expressed in the brain, including cortex and hippocampus (Schroeder *et al.*, 2000), and in skeletal muscle (Lerche *et al.*, 2000; Schroeder *et al.*, 2000). KCNQ5 currents activate slowly and biexponentially, and do not fully activate within 3 s of depolarization (Lerche *et al.*, 2000; Schroeder *et al.*, 2000). KCNQ5 current undergoes inward rectification at positive potentials, in a manner similar to KCNQ3 current (Schroeder *et al.*, 2000).

Heteromeric channels

Although KCNQ1 does not associate with other members of the KCNQ family (Schroeder *et al.*, 1998; Kubisch *et al.*, 1999; Lerche *et al.*, 2000), certain other members of KCNQ family can coassemble to form functional heteromeric K^+ channels.

KCNQ2 + KCNQ3 heteromeres produce a large current with properties similar to those of the neuronal "M-current" (Wang *et al.*, 1998; Main *et al.*, 2000). Coexpression of KCNQ2 and KCNQ3 increases the number of functional channels in the cell membrane compared to expression of KCNQ2 or KCNQ3 alone (Schwake *et al.*,

2000), and the A-domain is likely to play a role in subunit interaction (Lerche *et al.*, 2000; Schwake *et al.*, 2000).

KCNQ3 is unique in that it can form heteromeres with all members of KCNQ family, except for KCNQ1 (Schroeder *et al.*, 1998). KCNQ4 does not coassemble with KCNQ1 and KCNQ2 (Kubisch *et al.*, 1999). However, it can coassemble with KCNQ3, producing a large-amplitude current (Kubisch *et al.*, 1999). KCNQ5 interacts with KCNQ3, but probably not with KCNQ1, KCNQ2 or KCNQ4 (Lerche *et al.*, 2000; Schroeder *et al.*, 2000). Heteromeric KCNQ3 + KCNQ5 channels may be another physiological correlate of M-current (Lerche *et al.*, 2000; Schroeder *et al.*, 2000).

Role of β-subunits

A number of small, 1 TMD proteins can interact with channels of the KCNQ family and affect their biophysical and pharmacological properties. The first member of these auxiliary proteins, the 129 amino acid KCNE1 (formerly known as minK) was mapped to human chromosome 21 by Chevillard *et al.* (1993), and Splawski *et al.* (1998) determined the exon–intron structure of the gene. Coassembly of KCNQ1 with KCNE1 increases the KCNQ1 current amplitude, shifts activation to more positive potentials, and removes inactivation (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996; Tristani-Firouzi and Sanguinetti, 1998; Wang *et al.*, 1998). The resultant current has properties resembling those of cardiac I_{Ks}. The coassembly of KCNQ1 with KCNE1 also reverses the response of the resultant current to cytosolic pH changes and sensitivity to temperature compared to KCNQ1 current, and decreases sensitivity to channel blockers clotrimazole and tetrapentylammonium (Unsold *et al.*, 2000). KCNE1 interacts directly with the KCNQ1 channel pore, and residue F340 plays a

critical role in the channel subunit assembly (Melman *et al.*, 2004). Another two members of the KCNQ family that can coassemble with KCNE1 are KCNQ2 and KCNQ3 (Schroeder *et al.*, 1998; Yang *et al.*, 1998).

KCNE2, which associates with erg in the heart (Abbott *et al.*, 1999), is widely-distributed and can interact with both KCNQ2 homomeric channels and with KCNQ2 + KCNQ3 heteromeres (Tinel *et al.*, 2000). KCNE2 associates with KCNQ2 and KCNQ3, and accelerates the deactivation kinetics of the currents (Tinel *et al.*, 2000). KCNE2 affects properties of KCNQ1, at least in expression systems, producing a permanently active current that is characterized by rapid activation and deactivation, and a near-linear current–voltage (I–V) relationships (Tinel *et al.*, 2000).

KCNE3 was first cloned by similarity to KCNE1 (Abbott *et al.*, 1999). It is expressed in kidney, small intestine, colon, and heart. Association of KCNQ1 with KCNE3 produces current with near-instant activation, and linear I–V relationship (Schroeder *et al.*, 2000). When co-expressed with KCNQ4, KCNE3 drastically decreases the current amplitude (Schroeder *et al.*, 2000).

Grunnet *et al.* (2002) have cloned mouse KCNE4, which is expressed in a variety of tissues, including uterus, kidney, small intestine, heart, and lungs. Coexpression of KCNQ1 and KCNE4 almost completely inhibited the KCNQ1 current (Grunnet *et al.*, 2002) and slowed the activation time constant of KCNQ1 (Teng *et al.*, 2003).

Coexpression of KCNQ1 with KCNE5 shifts the activation by 140 mV to more positive potentials (Angelo *et al.*, 2002). KCNQ1 is the only member of the KCNQ family that is affected by coexpression with KCNE5 (Angelo *et al.*, 2002).

1.2.2 Physiological role

As mentioned above, the KCNQ1/KCNE1 protein complex produces a current with kinetics and voltage-dependence resembling those of cardiac I_{Ks}. Additional evidence that I_{Ks} is the physiological correlate of KCNQ1/KCNE1 current comes from the expression profile of KCNQ1 and KCNE1, and the similarity of I_{Ks} and KCNQ1/KCNE1 current in regard to sensitivity to channel blockers. Since several KCNE family proteins (KCNE1–KCNE5) are expressed in the heart, it is possible that the relative size and properties of cardiac I_{Ks} depend on the expression profile of different KCNE subunits (some of which can have an inhibitory effect on KCNQ1) (Lundquist *et al.*, 2005).

In the inner ear, mRNA for KCNQ1 and KCNE1 appears in the apical surface of marginal cells (Neyroud *et al.*, 1997). The KCNQ/KCNE1 channels are thought to be constitutively-active in the choclea and play a role in K^+ ion recycling.

KCNQ1 mRNA is also detected in intestine cells, where KCNQ1 coassembles with the KCNE3 auxiliary subunit (Schroeder *et al.*, 2000). The resultant current is instantaneous, with near-linear I–V relationship. The KCNQ1/KCNE3 current is inhibited by chromanol 293B and stimulated by intracellular cAMP. KCNQ1/KCNE3 recycles K^+ in the basolateral membrane of intestinal crypt cells, which is important for chloride secretion by these cells (Schroeder *et al.*, 2000).

M-current is a slowly activating, deactivating neuronal K^+ current that is inhibited by activation of G-protein coupled muscarinic receptors, and, therefore is termed "M-current". The current is blocked by linopirdine (Noda *et al.*, 1998) and XE991 (Wang *et al.*, 2000; Rennie *et al.*, 2001). The heteromeres of KCNQ2 + KCNQ3 are possible physiological correlates of M-current (Wang *et al.*, 1998; Cooper *et al.*, 2000). It is

possible, however, that additional subunits (for example, KCNE2) are required to generate M-type current (Tinel *et al.*, 2000).

The K^+ current recorded from the outer hair cells ($I_{K,\,n}$) has kinetics (Kubisch *et al.*, 1999; Kharkovets *et al.*, 2000), but not voltage-dependence (Mammano and Ashmore, 1996) or pharmacology (Marcotti and Kros, 1999) similar to that of KCNQ4 homomers or KCNQ3 + KCNQ4 heteromeres. The K^+ current ($I_{K,\,L}$) present in type I cells in the vestibular apparatus is also similar to KCNQ4 current (Kharkovets *et al.*, 2000).

1.3 Cardiac I_{Ks} and KCNQ1/KCNE1 channels

1.3.1 Biophysical properties

Cardiac delayed rectifier is a time- and voltage-dependent current that plays a role in myocyte repolarization. The current is comprised of two components: "rapidly-activated" I_{Kr} and "slowly-activated" I_{Ks}. These components can be separated by voltage protocols (I_{Kr} activates at more negative potentials than I_{Ks}), and by application of specific channel blockers (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a,b).

Activation of I_{Ks} in human and guinea pig ventricular myocytes is sigmoidal; when currents on depolarization to +50 mV were fitted with single exponentials, the time constants were 903 ms in human ventricular myocytes (Virag *et al.*, 2001) and 447 ms in guinea pig ventricular myocytes (Lu *et al.*, 2001). When fitted with double exponentials, activation of I_{Ks} in guinea pig ventricular myocytes pulsed to +50 mV had a fast activation time constant of 417 ms and a slow time constant of 1649

ms (Bosch *et al.*, 1998). Reported activation $V_{0.5}$ values in guinea pig ventricular myocytes range from +15.7 to +23.7 mV (Sanguinetti and Jurkiewicz, 1990; Fan and Hiraoka, 1991). The current does not inactivate during depolarizations, but deactivates on subsequent repolarization. When deactivating tail currents were fitted with monoexponential functions, the time constant of decay (τ) varied from 122 ms in human (Virag *et al.*, 2001) to 203 and 257 ms in guinea pig (Heath and Terrar, 1996b; Lu *et al.*, 2001) ventricular myocytes. In guinea pig ventricular myocytes, τ was near maximal (440 ms) at −20 mV, and declined at more negative and positive potentials (Matsuura *et al.*, 1987).

The single channel conductance of Ks channels was estimated to be ≈3 pS in a study on guinea pig atrial myocytes (Horie *et al.*, 1990). The coexpression of KCNE1 with KCNQ1 increases single channel conductance by 4–6 fold compared to the conductance of KCNQ1 alone (0.7 pS) (Yang and Sigworth, 1998).

In guinea pig ventricular myocytes, I_{Ks} has a Na^+/K^+ permeability ratio of 0.016 (Matsuura *et al.*, 1987). The channels are moderately permeable to Cs^+ (Cs^+/K^+ permeability ratio of ≈0.15), and highly permeable to Rb^+ (Hadley and Hume, 1990). I_{Ks} amplitude increases when extracellular potassium concentration ($[K^+]_o$) is lowered, consistent with an increase in the driving force for K^+ (Sanguinetti and Jurkiewicz, 1992). Removal of extracellular Ca^{2+} did not cause a loss of Ks channels K^+ selectivity (Sanguinetti and Jurkiewicz, 1992), unlike K^+ channels in squid neurons and expressed *Shaker* H4 K^+ channels (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990).

I_{Ks} is affected by the concentrations of intracellular cations. Williams and Beatch (1997) found that Mg^{2+} blocked I_{Ks} in guinea pig ventricular myocytes with an

EC_{50} of 24 nM. By contrast, elevation of $[Ca^{2+}]_i$ from 10 to 100 nM increased the amplitude of I_{Ks} by ≈3-fold without affecting the voltage-dependence of activation (Tohse, 1990). However, in a study of I_{Ks} in inside-out patches excised from guinea pig ventricular myocytes, an increase in $[Ca^{2+}]_i$ from 10 nM to 1 μM only increased the amplitude of the current by ≈60% (Nitta *et al.*, 1994). Increasing intracellular Na^+ to 65 mM blocked KCNQ1 and KCNQ1/KCNE1 channels expressed in CHO cells (Pusch *et al.*, 2001). In another study with Na^+, block of KCNQ1/KCNE1 channels expressed in COS cells was both voltage- and concentration-dependent, with dissociation constant of ≈300 mM (Orikabe *et al.*, 2003).

Native I_{Ks} is relatively insensitive to changes in extracellular pH (from 8.5 to 6.5) (Vereecke and Carmeliet, 2000), but there is no information on whether the current is affected by intracellular pH. Recombinant KCNQ1 and KCNQ1/KCNE1 channels are sensitive to changes in both extracellular and intracellular pH. Acidification of extracellular solution (pH 7.4 to pH 5.5) caused a rapid decline in KCNQ1 current amplitude, shifted current activation to more positive potentials, and slowed the kinetics of activation and deactivation (Peretz *et al.*, 2002). However, the effects of extracellular pH on KCNQ1/KCNE1 current were far less pronounced, with deletion of several amino acids on the N-terminal of KCNE1 drastically increasing current sensitivity to pH (Peretz *et al.*, 2002). Cytoplasmic acidification suppressed KCNQ1 current, but stimulated KCNQ1/KCNE1 current (Unsold *et al.*, 2000).

In guinea pig ventricular myocytes, I_{Ks} amplitude increases with an increase in bath solution temperature (temperature coefficient (Q_{10}) value of 3.8) (Walsh *et al.*, 1989). The increase in the temperature also accelerated current activation and deactivation (Walsh *et al.*, 1989). Homomeric KCNQ1 current is only slightly affected by

changes in temperature (Unsold *et al.*, 2000). In contrast, KCNQ1/KCNE1 channels expressed in CHO cells (Unsold *et al.*, 2000) and native KCNQ1/ expressed KCNE1 channels in *Xenopus* oocytes (Busch and Lang, 1993) are highly temperature-sensitive (Q_{10} values of 4–7).

1.3.2 Distribution

The density of I_{Ks} differs amongst species. The density is high in human (Wang *et al.*, 1994; Li *et al.*, 1996), guinea pig (Sanguinetti and Jurkiewicz, 1988, 1990; Turgeon *et al.*, 1994), and canine (Liu and Antzelevitch, 1995; Gintant, 1996) atrial and ventricular myocytes, but low in in feline ventricular myocytes (Follmer *et al.*, 1992), and in rabbit sino-atrial (Shibasaki, 1987; Lei and Brown, 1996), Purkinje fiber (Cordeiro *et al.*, 1998), and ventricular (Carmeliet, 1992; Clay *et al.*, 1995; Lu *et al.*, 2001) myocytes.

Relative I_{Ks} density also differs amongst myocyte subtypes within the ventricle. In dog, I_{Ks} density is higher in sub-epicardial and sub-endocardial myocytes than in mid-myocardial ventricular myocytes ("M-cells") (Liu and Antzelevitch, 1995), and higher in the right ventricle than in the left ventricle (Volders *et al.*, 1999). In guinea pig, I_{Ks} density in mid-myocardial and sub-epicardial myocytes is higher than that in sub-endocardial myocytes (Bryant *et al.*, 1998). In rabbit, I_{Ks} density is higher in sub-epicardial than in sub-endocardial myocytes (Xu *et al.*, 2001).

Within isolated rat ventricular myocytes, KCNQ1 is distributed in both T tubular membrane and peripheral sarcolemma (Rasmussen *et al.*, 2004).

1.3.3 Sensitivity to channel blockers and openers

I_{Ks} is selectively blocked by two chromanol compounds, 293B and HMR-1556 (Busch *et al.*, 1996; Gogelein *et al.*, 2000), as well as by indapamide (Turgeon *et al.*, 1994), propofol (Heath and Terrar, 1996a), thiopentone (Heath and Terrar, 1996a), and several benzodiazepines (L-168,673, L-735,821, L-7) (Lengyel *et al.*, 2001; Seebohm *et al.*, 2003). The block of I_{Ks} by chromanols is enantiomer-specific: in fact, (−)3R,4S-293B is a potent open-channel blocker. Similarly, benzodiazepine 3-S-L-364,373 (S-L3) inhibits I_{Ks}, whereas R-L3 is a channel opener (Salata *et al.*, 1998).

KCNE is known to modify the sensitivity of KCNQ1 to blockers: KCNQ1/KCNE1 channels are more sensitive to 293B, HMR-1556, and XE991 than KCNQ1 channels (Busch *et al.*, 1997; Selyanko *et al.*, 2000; Wang *et al.*, 2000). The molecular determinants of 293B and L-7 channel block are located in the pore region and S6 domain, as indicated by experiments on KCNQ1 and KCNQ2 chimeric constructs (Seebohm *et al.*, 2003).

Extracellular Ba^{2+} blocks homomeric KCNQ1 channels by a voltage-dependent mechanism that is characterized by a shift in the voltage dependence of activation to more positive potentials, slowed activation, accelerated deactivation, and inhibited channel inactivation (Gibor *et al.*, 2004).

Extracellular tetraethylammonium (TEA) blocks all types of KCNQ channels with EC_{50} values of 5, 0.3, >30, 3, and 70 mM for KCNQ1, KCNQ2, KCNQ3, KCNQ4, and KCNQ5, respectively (Hadley *et al.*, 2000; Schroeder *et al.*, 2000). The presence of tyrosine in the P region seems to confer high TEA sensitivity on KCNQ2 channels (Hadley *et al.*, 2000), a finding that is analogous to that on *Shaker* channels (Heginbotham and MacKinnon, 1992).

Mefenamic acid (100 μM) and DIDS (100 μM) activate KCNQ1/KCNE1 channels, but have negligible effects on KCNQ1 channels (Busch *et al.*, 1997). DIDS shifts the voltage-dependence of activation to the left and slows deactivation of the current (Abitbol *et al.*, 1999). Mefenamic acid and DIDS bind to the extracellular N-terminal segment on KCNE1 (Busch *et al.*, 1997) and restore gating of inactive C-terminal KCNE1 mutants (Abitbol *et al.*, 1999). KCNE1 also determines the sensitivity of KCNQ1/KCNE1 channels to stimulation by fatty acids (Doolan *et al.*, 2002).

1.3.4 Regulation by cell signaling mechanisms

Protein kinase A

Activation of β-adrenoreceptors causes an exchange of GDP for GTP on G_s type G-proteins and their dissociation into $G_{s\alpha}$ and $G_{s\beta\gamma}$ subunits. $G_{s\alpha}$ stimulates adenylate cyclase (AC) which, in turn, increases production of 3',5'-cyclic monophosphate (cAMP). An increased cytoplasmic concentration of cAMP stimulates cAMP-dependent protein kinase A (PKA), a kinase that phosphorylates a multitude of target proteins, including ion channels and pumps. It has long been known that β-adrenergic stimulation increases the force of contraction and frequency of the heartbeat, as well as the rate of relaxation after heart muscle contraction (Hartzell, 1988). The latter effect can be in part be attributed to PKA-dependent stimulation of cardiac delayed-rectifier K^+ current (Walsh and Kass, 1988; Harvey and Hume, 1989; Yazawa and Kameyama, 1990). An elevation of PKA activity causes a -20 mV shift in the activation relationship of I_{Ks}, increases the current amplitude by 2–3 fold, and slows deactivation of I_{Ks} in guinea pig ventricular myocytes (Walsh and Kass, 1991) and endogeneous KCNQ1/ expressed KCNE1 current in *Xenopus* oocytes (Lo and Numann,

1998).

Recently, scaffolding protein yotiao (AKAP1) has been implicated in the recruitment of PKA and protein phosphatase type 1 (PP1) into a tightly-coupled signaling system with KCNQ1/KCNE1 channels that potentiates of channel PKA phosphorylation (Marx *et al.*, 2002). Serine S27 located on the N-terminus of KCNQ1 is a putative PKA phosphorylation site (Marx *et al.*, 2002). Mutation S27A, disruption of the leucine zipper motif on the C-end of KCNQ1, or expression of KCNQ1/KCNE1 channels in cells that lack yotiao results in channels that are insensitive to increases in the concentration of intracellular cAMP (Marx *et al.*, 2002). In addition, yotiao itself can be PKA-phosphorylated on serine 43, and mutations affecting a yotiao PKA phosphorylation site abolish the responses of KCNQ1/KCNE1 current to elevated cAMP concentration and to application of PKA catalytic subunit (Chen *et al.*, 2005).

Protein kinase C

Stimulation of serine/threonine protein kinase C (PKC) enhances I_{Ks} in guinea pig ventricular myocytes (Tohse *et al.*, 1987; Walsh and Kass, 1988; Tohse *et al.*, 1990; Yazawa and Kameyama, 1990; Asai *et al.*, 1996). The degree of I_{Ks} stimulation by PKC is generally weaker than that by PKA (≈30–50% increase in current amplitude) (Tohse *et al.*, 1990; Yazawa and Kameyama, 1990). Unlike stimulation by PKA, PKC stimulation of I_{Ks} does not change the $V_{0.5}$ of activation, but increases the slope of the activation relationship (Walsh and Kass, 1991). The stimulation of I_{Ks} by PKC required $[Ca^{2+}]_i$ higher than 10^{-10} M (Tohse *et al.*, 1987), and dependence of I_{Ks} amplitude on $[Ca^{2+}]_i$ was shifted to the left by PKC activator 2-O-tetradecanoylphorbol-13-acetate (TPA) (Tohse *et al.*, 1990), suggesting a $[Ca^{2+}]_i$ dependent mechanism of

I_{Ks} stimulation by PKC.

Current carried by endogeneous KCNQ1/ expressed KCNE1 in *Xenopus* oocytes was stimulated by PKC, but the time course of the effect was considerably slower than in studies on native I_{Ks} (Zhang *et al.*, 1994).

Protein kinase G

Intracellular dialysis of guinea pig sino-atrial myocytes with 100 μM cGMP caused an increase in the amplitude of I_{Ks}, possibly via inhibition of cGMP-inhibited phosphodiesterase 3 (PDE3) and stimulation of protein kinase G (PKG) (Shimizu *et al.*, 2002).

G-protein coupled receptors

As described above, β-adrenergic stimulation augments I_{Ks} by activation of G_s type G-proteins and subsequent stimulation of PKA. However, $G_{s\alpha}$ can also stimulate I_{Ks} via a membrane-delimited pathway (Freeman *et al.*, 1992). PKA-stimulated I_{Ks} is inhibited by muscarinic receptor-activated G_i type G-proteins that downregulate AC (Harvey and Hume, 1989).

Stimulation of KCNQ1/KCNE1 channels by activation of β_3 -adrenoreceptors expressed in *Xenopus* oocytes was mediated by PKC and diminished by mutations in the putative PKC phosphorylation sites on KCNQ1 (Kathofer *et al.*, 2003).

I_{Ks} is positively regulated by G_q-coupled P2Y receptors, and this regulation is likely to involve a tyrosine kinase-mediated step (Matsubayashi *et al.*, 1999). The α_1-adrenoreceptor-mediated increase in the amplitude of I_{Ks} in guinea pig ventricular myocytes could be related to stimulation of PKC (Tohse *et al.*, 1992).

Endothelin, a vasoconstricting peptide, stimulated delayed rectifier potassium current I_{Ks} in guinea pig ventricular myocytes by activating G-protein coupled endothelin receptors, with possible resultant stimulation of PKC (Habuchi *et al.*, 1992). In another study (Washizuka *et al.*, 1997), endothelin had an early inhibitory action (possibly via inhibition of AC by pertussis toxin (PTX)-sensitive G-protein), and a late PKC-dependent stimulatory action on I_{Ks}. When KCNQ1/KCNE1 current in *Xenopus* oocytes was maximally stimulated by β-adrenoreceptor agonist isoproterenol, endothelin inhibited the current instead of stimulating it as under control conditions, suggesting crosstalk between β-adrenoreceptor and ET-A receptor signaling (Lin *et al.*, 2005).

The increase of I_K amplitude in guinea pig ventricular myocytes caused by application of histamine has been attributed to an increase in cyclic AMP-dependent phosphorylation (Yazawa and Abiko, 1993). More recently, the increase in I_{Ks} in guinea pig *atrial* myocytes has been attributed to a stimulation of PKC (Matsumoto *et al.*, 1999).

Phosphatidylinositol 4,5-bisphosphate

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is membrane phospholipid involved in many signaling pathways. PIP_2 is produced by consecutive phosphorylation of the hydroxyl groups on phosphatidylinositol (PI). PIP_2 is a substrate for further phosphorylation and cleavage by phosphatidylinositol-3-kinase (PI3-K) and for hydrolysis by phospholipase C (PLC). Application of a PI3-K and phosphatidylinositol-4-kinase (PI4-K) inhibitor wortmannin (50 μM) stimulated I_{Ks} in guinea pig atrial myocytes,

an effect that was attributed to a decline in PIP_2 concentration. In addition, intracellular application of 100 μM PIP_2 inhibited the current (Ding *et al.*, 2004). On the other hand, application of PIP_2 to patches excised from COS-7 cells slowed the rundown of KCNQ1/KCNE1 current, suggesting stabilization of the open state (Loussouarn *et al.*, 2003). PIP_2 also stimulated KCNQ1/KCNE1 current in inside-out patches excised from *Xenopus* oocytes (Zhang *et al.*, 2003), and counterbalanced loss of function mutations (R243H, R539W, and R555C) in KCNQ1 channels (Park *et al.*, 2005).

Tyrosine kinase

In canine ventricular myocytes, a 50-μM concentration of broadspectrum protein tyrosine kinase (PTK) inhibitor genistein decreased the amplitude of I_{Ks} by 46%, suggesting that PTK has a positive regulatory influence on the current (Zhou *et al.*, 1997). However, Washizuka *et al.* (1997, 1998) concluded that inhibition of I_{Ks} by genistein was due to a direct blocking effect on Ks channels in guinea pig ventricular myocytes. In regard to PTK and expressed KCNQ1 channels, overexpression of Src PTK did not result in detectable tyrosine phosphorylation of KCNQ1 channels (Gamper *et al.*, 2003; Li *et al.*, 2004).

Several putative tyrosine phosphorylation sites exist on KCNQ1 protein: Y51 and Y111 on the N-end of the protein, Y171 and Y184 on the intracellular loop between TMDs S2 and S3, and Y395, Y461, and Y662 on the C-end of KCNQ1. The exact role of these tyrosine residues in modulation of the properties of KCNQ1 current is unclear. However, it appears that Y111 is important for the membrane localization

of KCNQ1: when this residue was mutated to alanine, channels expressed in Madin-Darby canine kidney (MDCK) cells were localized in the cytoplasm, and no KCNQ1 current could be recorded (Jespersen *et al.*, 2004). Mutation of residue Y51 caused a non-polarized membrane distribution of KCNQ1 protein, whereas expressed wild type KCNQ1 is localized in the basolateral membrane of MDCK cells (Jespersen *et al.*, 2004). The putative tyrosine phosphorylation site on KCNE1 is Y94. No information is available on the role of this residue in the modulation of KCNQ1/KCNE1 channels, however, deletion of residues 94–129 in KCNE1 did not impair the ability of KCNE1 to modulate KCNQ1 gating (Tapper and George, 2000).

1.3.5 Role in cardiac repolarization

Because I_{Ks} activates slowly after a pronounced delay, the current amplitude reaches its maximum during the late stages of the action potential plateau phase (Zeng *et al.*, 1995). The activation of I_{Ks} increases the net outward current and thereby assists in repolarization of the myocyte membrane. The pharmacological block of I_{Ks} in guinea pig and human ventricular myocytes by 1 μM 293B caused a prolongation of the action potential duration (APD) (Bosch *et al.*, 1998). The relative increase in APD was similar in the two species (30–40%). In myocyte types that have lower density of I_{Ks}, for example, in canine (Varro *et al.*, 2000) and rabbit (Lengyel *et al.*, 2001) ventricular myocytes, block of I_{Ks} with chromanol 293B (10 μM) and L-735,821 (100 nM) did not significantly affect APD. At high pacing rates (Lu *et al.*, 2001) and under conditions of β-adrenergic stimulation of I_{Ks} (Stengl *et al.*, 2003), the current "accumulates" due to the incomplete deactivation. This accumulation shortens the APD.

1.3.6 Pathology

"Long QT" is a form of cardiac disorder characterized by an increased interval between Q and T peaks on the electrocardiogram (ECG). The prolonged QT reflects a delay in ventricular repolarization, and can be both acquired and inherited. Several forms of congenital long QT (LQT) syndrome have been described, and, with the exception of LQT4, they are related to mutations in genes encoding ion channel proteins or their auxiliary subunits.

The most common form of LQT, LQT1, is related to the impaired function of KCNQ1. Wang *et al.* (1996) found KCNQ1 mutations in affected members of 16 families with long QT syndrome 1. Subsequently, Shalaby *et al.* (1997) found that mutations in the KCNQ1 gene that mimicked the data obtained by Wang *et al.* (1996) yielded either non-functional channels, or channels with a greatly reduced conductance. An autosomal dominant form of LQT1 (Romano-Ward (RW) syndrome) was reported independently in 1963 and 1964 by Romano et al. (Romano *et al.*, 1963a,b) and Ward (Ward, 1964). The autosomal recessive cardioauditory syndrome was reported in 1957 by Jervell and Lange-Neilsen (Jervell and Lange-Nielsen, 1957), and is characterized by weaker (compared to RW) cardiac dysfunction and (in the homozygous form) by inherited deafness.

Neyroud *et al.* (1997) used homozygosity mapping to locate a gene for Jervell and Lange-Neilsen cardioauditory syndrome. They found it to be located in the region 11p15.5, similar to KCNQ1. An animal model for Jervell and Lange-Nielsen syndrome was created by Casimiro *et al.* (2001). The homozygous mice were deaf and ECG recorded from null mice demonstrated abnormal T- and P-wave morphologies and prolongation of QT and JT intervals.

Recently, a gain-of-function mutation (S140G) in KCNQ1 has been implicated in cases of familial atrial fibrillation (Chen *et al.*, 2003). The mutation causes an increase in the amplitude of atrial I_{Ks}, which shortens the APD and is likely to promote reentrant atrial arrhythmia (Chen *et al.*, 2003).

Mutations in the KCNE1 subunit were identified in patients with LQT5 syndrome (Duggal *et al.*, 1998; Abitbol *et al.*, 1999). The coassembly of mutated KCNE1 subunits with KCNQ1 suppressed channel function and increased the rate of channel deactivation.

1.4 Responses of cardiac myocytes to osmotic stress

1.4.1 Mechanism of osmosis

Cell membranes are more permeable to water than to ions. For Ehrlich epithelial cells, the membrane water permeability exceeds K^+ permeability by a factor of 25,000 (Hoffmann *et al.*, 1983). Even though the lipid bilayer itself is permeable to water, in living cells water crosses the membrane via "water channels" termed aquaporins (Agre *et al.*, 1993).

Addition of an osmolyte to either the extracellular or intracellular solution changes water concentration in that solution and creates a water concentration gradient across the cell membrane. The osmotic pressure (i.e., the pressure on the cell membrane in response to an osmotic gradient) is proportional to the difference in the molecule concentration between the intracellular and extracellular solution (van't Hoff law):

$$\pi = RT \sum_n c_n \tag{1.1}$$

where π is osmotic pressure, R the universal gas constant, T the absolute temperature, and $\sum c$ the sum of osmolyte concentrations.

Animal cells are surrounded by a thin and flexible membrane, and cannot withstand high osmotic pressure. As with other cells, exposure of cardiac myocytes to an anisosmotic bathing solution (a solution that has a different total concentration of ions and macromolecules (osmolytes) than the cytoplasm) causes a rapid swelling or shrinkage of the myocyte. When exposed to anisosmotic solution, cells (including cardiac myocytes) behave as "perfect osmometers", i.e., their volume changes linearly with the difference in osmolyte concentration on the two sides of the cell membrane (Pine *et al.*, 1981; Drewnowska and Baumgarten, 1991).

The presence of large organic molecules inside the cell creates an osmotic gradient which results in a constant tendency for cells to take up water and swell. The active pumping of Na^+ out of the cell by the Na^+–K^+ pump counterbalances this osmotic imbalance. In addition, if the osmolarity of the extracellular solution decreases, water will enter the cell following the osmotic gradient, and the cell will swell. Many types of cells are able to respond to the swelling by undergoing a secondary compensatory shrinkage, called a Regulatory Volume Decrease (RVD). RVD is achieved by the efflux of ions through K^+ and Cl^- channels, and by activation of the K^+/Cl^- symporter until osmotic equilibrium is reached (Hoffmann *et al.*, 1983; Hoffmann, 1985, 2001). When cells are exposed to hyperosmotic extracellular solution, they may undergo a Regulatory Volume Increase (RVI).

1.4.2 Cardiac myocyte volume regulation

Cardiac myocytes are unusual in their volume responses. First, the membrane of cardiac myocytes is less permeable to water than the membrane of many other cell types (possibly due to the lower density or activity of aquaporins) (Suleymanian and Baumgarten, 1996). Nevertheless, up to 80% of water transport across the membrane of guinea pig ventricular myocytes is likely to be through aquaporins (Ogura *et al.*, 2002). Second, in the absence of other interventions, cardiac myocytes do not undergo pronounced RVD or RVI (Drewnowska and Baumgarten, 1991) (however, stimulation of PKA activity causes RVD in cultured chick cardiac myocytes (Zhang *et al.*, 1993, 1997) and in guinea pig ventricular myocytes (Wang *et al.*, 1997)). Third, blockade of the Na^+–K^+ pump with ouabain did not affect rabbit ventricular myocyte volume under isosmotic conditions (Drewnowska and Baumgarten, 1991), whereas in many other cell types such a block causes cell swelling. On the other hand, response to swelling was increased by 76% and response to shrinkage was decreased by 29% in myocytes pretreated with blocker of Na^+–K^+–Cl^- co-transporter bumetanide and blocker of Na^+–Cl^- co-transporter chlorothiazide (Drewnowska and Baumgarten, 1991).

When do cardiac myocytes face changes in extracellular osmolarity? Under ischaemic conditions, cardiac myocytes accumulate osmolytes (such as lactate) due to the switch to the anaerobic metabolism. In the reversible phase of ischaemia myocyte lactate concentration increases by 48 mM, and in the irreversible phase the accumulation of up to 96 mM is possible (Jennings *et al.*, 1986). The accumulation of lactate is expected to cause myocyte swelling when concentration of osmolytes outside of myocyte is lower due to the slow diffusion or washout. In addition, when normal flow

of extracellular fluid is restored (reperfusion after ischaemic episode), the extracellular osmolyte concentration rapidly decreases, causing severe, "explosive" myocyte swelling (Garcia-Dorado and Oliveras, 1993).

Hyponatremia, defined as a decrease of blood plasma sodium concentration below 130 mM, is a frequent electrolyte disorder (Berry and Belsha, 1990). Hyponatremia can be caused by a variety of factors, including elevated water uptake ("water poisoning") or retention, lowered sodium uptake, and increased sodium loss. In severe cases, serum sodium concentration can decrease below 100 mM (Gross *et al.*, 2001).

Hyperosmotic intravenous fluids are used for the volume support and resuscitation of hemorrhage and trauma (Kramer, 2003). Such a mode of therapy may cause an osmolyte disbalance and lead to cardiac myocyte swelling or shrinkage.

Effects of cell osmolarity on ionic currents, pumps, and exchangers in cardiac myocytes

Even though cardiac myocytes do not exhibit a pronounced RVD or RVI, a change in external osmolarity modulates a number of channel and pump currents, including volume-sensitive Cl^- current ($I_{Cl,\,vol}$) (Tseng, 1992; Shuba *et al.*, 1996; Du and Sorota, 1997; Clemo *et al.*, 1998), ATP-dependent K^+ current ($I_{K,\,ATP}$) (Priebe and Beuckelmann, 1998), inward-rectifier K^+ current (I_{K1}) (Missan *et al.*, 2004), L-type Ca^{2+} current (Matsuda *et al.*, 1996; Ogura *et al.*, 1997; Kim *et al.*, 2000; Kimura *et al.*, 2000), T-type Ca^{2+} current (Pascarel *et al.*, 2001), nonselective cation channel current (Kim and Fu, 1993; Clemo and Baumgarten, 1997), Na^+-Ca^{2+} exchanger current (Wright *et al.*, 1995; Ogura *et al.*, 1997), and Na^+-K^+ pump current (Whalley *et al.*, 1993; Sasaki *et al.*, 1994; Bewick *et al.*, 1999).

Sasaki *et al.* (1992, 1994) reported that extracellular hyposmotic solutions stimulate, and hyperosmotic solutions suppress, I_{Ks} in guinea pig ventricular myocytes. Modulatory effects of external anisosmotic solutions have also been shown for canine cardiac I_{Ks} (Zhou *et al.*, 1997), guinea pig cardiac I_{Ks} (Rees *et al.*, 1995; Groh *et al.*, 1996; Kocic *et al.*, 2001; Ogura *et al.*, 2003), KCNQ1 and KCNQ1/KCNE1 currents in COS-7 cells (Kubota *et al.*, 2002), and KCNQ1/KCNE1 current in *Xenopus* oocytes (Grunnet *et al.*, 2003). Application of extracellular hyposmotic solution also stimulates KCNQ4 (Grunnet *et al.*, 2003) and KCNQ5 (Jensen *et al.*, 2005) channels, but not KCNQ2 and KCNQ3 channels (Grunnet *et al.*, 2003).

1.4.3 Role of tyrosine kinase and phosphatase in the osmotic response

PTK

PTK is a class of enzymes that catalyze the transfer of the γ-phosphate from ATP to the hydroxyl groups of tyrosines on target proteins (Hunter, 2002). PTKs are involved in the regulation of a number of cellular processes, including cell growth and proliferation, differentiation, anti-apoptotic regulation, migration, and metabolism. As a result of the Human genome project, 90 putative PTKs have been identified (Blume-Jensen and Hunter, 2001). PTKs in the human genome can be subdivided into the two major groups: 58 members of the receptor tyrosine kinase (RTK) group that are subdivided into 20 families; and 32 members of the cytoplasmic, non-receptor tyrosine kinase group, comprised of 10 families.

RTKs are integral membrane proteins, many of which posses an intrinsic tyrosine kinase activity. Upon binding of the ligand to the extracellular domain of RTK,

the receptor undergoes dimerization and subsequent autophosphorylation; these reconfigurations lead to the formation of docking sites for intracellular signaling transducers.

Cytoplasmic PTKs are located in the cytoplasm, nucleus, and mitochondria, and can bind to the cell membrane (Hantschel and Superti-Furga, 2004). These kinases play a role in a variety of signaling pathways and processes, including mitogenesis, cytoskeleton reorganization, exocytosis, endocytosis, and ion channel regulation.

RTKs such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), and cytoplasmic PTKs such as Src and Fyn, are expressed and active in cardiac myocytes. PTKs are involved in modulation of the activity of many types of ion channels, including positive and negative regulation of voltage-gated K^+ channels (for review, see Davis *et al.*, 2001). For example, recombinant Kv1.2 (Tsai *et al.*, 1999), Kv1.3 (Holmes *et al.*, 1996b; Bowlby *et al.*, 1997), and Kv1.5 (Holmes *et al.*, 1996a) currents are suppressed by an increase in PTK activity, whereas native Kv channels in mouse Schwann cells are stimulated by Src-family PTK $p55^{fyn}$ (Sobko *et al.*, 1998), and both Kv1.5 and Kv2.1 are constitutively activated by tyrosine phosphorylation in these cells, with Fyn directly interacting with Kv channel subunits (Sobko *et al.*, 1998; Peretz *et al.*, 1999). Rat erg1 channels have also been shown to be positively regulated by Src phosphorylation of channel protein (Cayabyab and Schlichter, 2002).

Both RTK and cytoplasmic PTK are involved in cell responses to osmotic and mechanical stress. Studies performed with broadspectrum PTK inhibitors (such as genistein, PTK-active tyrphostins, and lavendustin A) indicate that they modulate

swelling- and stretch-activated currents in both cardiac and non-cardiac cells. For example, I_{Ks} in canine ventricular myocytes (Zhou *et al.*, 1997), Ba^{2+} current through L-type Ca^{2+} channels (I_{Ba}) in canine basilar arterial myocytes, and $I_{Cl,\,vol}$ in cultured astrocytes (Crepel *et al.*, 1998), calf pulmonary artery endothelial cells (Voets *et al.*, 1998), mouse fibroblasts (Bryan-Sisneros *et al.*, 2000), and dog atrial myocytes (Sorota, 1995) are inhibited by application of PTK inhibitors. The EGFR inhibitor tyrphostin AG1478 and EGFR/JAK2 inhibitor B42, but not the PDGFR inhibitor AG1295, suppressed extracellular receptor kinase (ERK) activation by stretch in mice cardiac myocytes (Kudoh *et al.*, 1998). Hypotonic stress caused a rapid activation of EGFR and subsequent activation of mitogen-activated protein kinase (MAPK) cascade in cultured rat cardiac myocytes (Sadoshima *et al.*, 1996). A direct mechanical stimulation by inflation of rabbit ventricular myocytes stimulates $I_{Cl,\,vol}$ via an Src-dependent pathway (Browe and Baumgarten, 2003), and $I_{Cl,\,vol}$ in both atrial and ventricular rabbit myocytes is stimulated by genistein (Du *et al.*, 2004; Ren and Baumgarten, 2005).

Phosphotyrosyl phosphatase

Tyrosine dephosphorylation, the opposite of tyrosine phosphorylation, is carried out by phosphotyrosyl phosphatase (PTP). The human genome contains approximately 100 types of PTP, which can be further subdivided into classical PTP, and dual-specificity PTP that can dephosphorylate both phosphotyrosine and serine/threonine residues.

Classical PTP are subdivided into receptor PTP and intracellular PTP (for review, see Zhang *et al.*, 2002). Similar to RTKs, receptor PTPs (for example, CD45) are

transmembrane proteins that contain an extracellular ligand-binding domain, whereas intracellular PTPs (for example, PTP1B and SHP1) are localized in plasma membrane and endoplasmic reticulum. All PTPs are characterized by their sensitivity to inhibition by vanadate (Swarup *et al.*, 1982; Gordon, 1991; Zhang *et al.*, 2002).

Little is known about the involvement of PTP in the response of cells to anisosmotic stress. In budding yeast, PTP2 and PTP3 are involved in the negative regulation of the osmotic stress-activated HOG pathway (Jacoby *et al.*, 1997). $I_{\text{Cl, swell}}$ in human and rabbit ventricular myocytes was modestly inhibited by application of a broadspectrum PTP inhibitor orthovanadate (Ren and Baumgarten, 2005), and pervanadate inhibited $I_{\text{Cl, swell}}$ in mouse L-fibroblasts (Thoroed *et al.*, 1999) and in bovine chromaffin cells (Doroshenko, 1998). On the other hand, application of orthovanadate neither affected KCNQ1 channels expressed in COS-7 cells under basal conditions nor prevented a decrease in current amplitude upon reversal of hyposmotic stimulation of KCNQ1 current (Kubota *et al.*, 2002).

1.5 Objectives of the study

The primary objective of the present study was to elucidate the role of tyrosine phosphorylation/dephosphorylation in stimulation of I_{Ks} by hyposmotic solution. The study used patch-clamp of guinea pig ventricular myocytes and utilized a broad range of PTK and PTP inhibitors to determine the degree of regulation of I_{Ks} by PTK/PTP. In order to approach the main objective, it was also necessary to obtain more information on the regulation of I_{Ks} by PTK and PTP under isosmotic conditions.

As mentioned above, cardiac I_{Ks} is modulated by several kinase and second messengers, including PKA, PKC, G-proteins, and PIP_2. The role of these and other

pertinent signaling molecules in the stimulation of I_{Ks} by hyposmotic solution had to be carefully examined.

I_{Ks} stimulation by hyposmotic solution ultimately affects cardiac myocyte repolarization. The present study tested how change in the amplitude of I_{Ks} stimulated by hyposmotic stress modulates the properties of simulated cardiac action potentials.

2

Methods

2.1 General experimental methods and procedures

2.1.1 Preparation of myocytes

Male guinea pigs (250–300 g) were killed by cervical dislocation, and single ventricular myocytes were isolated using an enzymatic dissociation method. The excised hearts were mounted on a Langendorff column and perfused through the aorta with Ca^{2+}-free Tyrode's solution containing collagenase (0.08–0.12 mg/ml; Yakult Pharmaceutical, Tokyo, Japan) for 10–15 min. All perfusates were oxygenated and warmed to 37°C. The ventricles were cut into small pieces, and cells were dispersed by mechanical agitation. The cell suspension was filtered through a nylon mesh and isolated cells were kept in a storage solution that contained (in mM) KOH 80, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, glutamic acid 50, taurine 20, ethylene glycol-bis(b -aminoethyl ether) -N,N,N,N-tetraacetic acid (EGTA) 0.5, and N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid (HEPES) 10 (pH 7.4 with KOH) (temperature 22°C).

A small aliquot of the cell suspension was placed in a 0.3-ml perfusion chamber mounted on the stage of an inverted microscope. After cells had attached to the bottom of the chamber, the chamber was perfused (≈2 ml/min) with Tyrode's solution warmed to 36°C using a custom-made Peltier heating device.

2.1.2 Composition of solutions

External solutions

The normal Tyrode's solution used to superfuse myocytes contained (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, and HEPES 5 (pH 7.4 with NaOH). The control isosmotic (1T) solution that was used to superfuse myocytes in experiments with anisosmotic solutions was a 50% Na^+ (70 mM NaCl) K^+-, Ca^{2+}-free Tyrode's solution (KCl and $CaCl_2$ omitted) that was supplemented with 140 mM D-mannitol (Sigma-Aldrich, St. Louis, MO, USA), and normally contained 0.2 mM Cd^{2+}; the osmolarity of the 1T solution was 293 mosm/l when measured using the freezing-point depression method (Ogura *et al.*, 1997). Anisosmotic solutions were obtained by varying the D-mannitol concentration in the 50% Na^+ Tyrode's solution; for example, 70 mM was added to make 0.75T hyposmotic solution, and 210 mM was added to make 1.25T hyperosmotic solution. In some experiments, 1T and other solutions contained 5.4 mM KCl .

The Ca^{2+}-free Tyrode's solution used for cell isolation contained (in mM): NaCl 125, KCl 4.6, $MgCl_2$ 1.15, taurine 20, glucose 20, and HEPES 5 (pH 7.4 with NaOH). Storage solution contained (in mM): KCl 30, KOH 80, KH_2PO_4 30, glutamic acid 50, $MgSO_4$ 3, taurine 20, glucose 10, EGTA 0.5, and HEPES 10 (pH 7.4 with KOH).

Pipette-filling solutions

The standard pipette-filling solution contained (in mM) KCl 40, potassium aspartate 106, $MgCl_2$ 4.8, K_2-ATP 4, Mg-ATP 1, EGTA 5, and HEPES 5 (pH 7.2 with KOH). The free Mg^{2+} and Ca^{2+} concentrations of this solution calculated with the Fabiato program (Fabiato and Fabiato, 1979) were 0.89 mM and <1 nM, respectively.

2.1.3 Chemicals and drugs

All chemicals used to make solutions were purchased from Sigma and were of the highest purity grade available.

Tyrphostins A25, A23, A1, A63, and AG1478 were supplied by Calbiochem (La-Jolla, CA, USA), dissolved in dimethyl sulfoxide (DMSO) (Sigma), and stored as 0.1 M stock solutions. The stock solutions were kept in the dark at −20°C, and superfusates containing the drugs were protected from light during all experiments. Bis(N,N-dimethylhydroxamido) hydroxooxovanadate (DMHV) (Calbiochem) was prepared as a 0.1 M stock solution and was stored at −70°C. Genistein, genistin, and daidzein (Sigma) were prepared as 100 mM stock solutions in DMSO and protected from light during the experiments. 1-(5-Isoquinolinesulfonyl) -2-methylpiperazine (H7), N-[2 -(Methylamino)ethyl] -5 -isoquinolinesulfonamide (H8), and N-[2-((p -Bromocinnamyl)amino)ethyl] -5-isoquinolinesulfonamide (H89) were obtained from Calbiochem, prepared as stock solutions in DMSO and kept frozen at −20°C. Isoproterenol (ISO) (Calbiochem) was prepared as an aqueous stock solution containing 1 mM ascorbic acid to prevent drug oxidation. Acetylcholine (ACh) (Calbiochem), human angiotensin II (Calbiochem), and NaATP (Sigma) were prepared as aqueous

stock solutions. Aqueous solutions of sodium orthovanadate (Van) (Fisher Scientific, Nepeon, ON, Canada) were prepared freshly before experiments, and the pH of orthovanadate-containing solutions was adjusted to 7.4 with HCl. E4031 (Eisai, Tokyo, Japan), chromanol 293B (Aventis, Strasbourg, France), and cytochalasin D (Sigma) were directly dissolved in bathing solutions. Glibenclamide (Sigma), 4-amino-5-(4-chlorophenyl) -7-(t-butyl) pyrazolo[3,4 -D]pyrimidine (PP2), 4-amino-7-phenylpyrazol[3,4- D]pyrimidine (PP3) (Calbiochem), bisindolylmaleimide I (Bis) (Calbiochem), phorbol-12-myristate-13-acetate (PMA) (Calbiochem), forskolin (FSK) (Calbiochem), and LY294002 (Calbiochem) were prepared as stock solutions in DMSO, kept at $-20°C$ and added to superfusates prior to experiments. PD98059 (Calbiochem) was dissolved in DMSO and added to both storage and pipette-filling solutions. GDPβS was dissolved in distilled water and added to pipette-filling solutions. The final concentration of DMSO in external solutions was $\leq 0.1\%$.

2.1.4 Electrophysiological recordings

Whole-cell membrane currents were recorded using an EPC-9 amplifier (HEKA Electronics, Mahone Bay, NS, Canada). Recording pipettes were fabricated from thick-walled borosilicate glass capillary (H15/10/137, Jencons Scientific, Leighton Buzzard, UK), and had resistances of 1.5–2.5 MΩ when filled with pipette solution. Pipette offsets were nulled prior to patch formation and liquid junction potentials (≈ -11 mV for the normal Tyrode's solution–K^+ pipette solution combination) were compensated for (-10 mV) during data analysis. Series resistances ranged between 3 and 5 MΩ and were compensated by 60–80%. Myocyte membrane capacitance (C_m) ranged between 110 and 140 pF. Membrane currents were filtered at 3 kHz and digitized with Pulse

software (HEKA Electronics) at a sampling rate of 12 kHz. Data files were converted from Pulse to Axon (Union City, CA, USA) data format, and analyzed with Axon Clampfit electrophysiology software.

2.1.5 Expressed KCNQ1/KCNE1 channels

Baby hamster kidney (BHK) cells were cotransfected with wild-type human KCNQ1, human KCNE1, and green fluorescence protein (GFP)-containing pIRES vectors using Lipofectamine (Invitrogen Canada Inc., Burlington, ON, Canada) reagent protocol. The cells were transfected and maintained by personnel in Dr. Paul Linsdell's laboratory. The efficiency of expression was assessed by the intensity of cell fluorescence at 470 nm excitation wavelength. The standard whole-cell patch clamp technique (see Section 2.1.4 above) was used to record membrane currents. In brief, small pieces of coverslip glass containing transfected cells were transferred into the recording bath. Both extracellular and pipette solutions had the same composition as those used in experiments on myocytes. Experiments were performed 24–72 hours after transfections at room temperature.

2.1.6 Myocyte volume measurement

To estimate changes in myocyte dimensions, isolated myocytes were placed in a perfusion chamber mounted on the stage of an inverted microscope. The cell images were recorded using a video system (series 67 camera system, Dage-MTI, Michigan City, IN, USA), and cell width and length were measured offline as described previously (Ogura *et al.*, 1995).

Myocyte volume was calculated from surface dimensions as $volume = length \times$

$width^2$, assuming that changes in cell width and thickness under anisosmotic conditions are proportional (Drewnowska and Baumgarten, 1991; Ogura *et al.*, 1995). All experiments were carried out at 36°C.

2.1.7 Computer simulations

Simulations were preformed using Cell Electrophysiology Simulation Environment (CESE) v. 1.3.3 (Missan and McDonald, 2005) using the Luo–Rudy phase II model of cardiac electrical activity (Luo and Rudy, 1994a,b; Faber and Rudy, 2000). A fixed step-size of 0.05 ms was used for all simulations.

2.1.8 Data analysis and statistics

Results are expressed as means ± SEM, and statistical comparisons were made using Student's paired or unpaired t-test. Differences were considered significant when $p < 0.05$.

Because of the relative consistency of C_m values, the magnitude of currents were expressed as current amplitude, rather than current density. When possible, relative change in the current amplitude was used for the statistical analysis.

Concentration–current (I) response relationships were fitted to the Hill equation (2.1), where $[X]$ is concentration of drug X, EC_{50} is the concentration that causes half-maximal response, and n_H is a Hill coefficient.

$$I = I_{max} \frac{[X]^{n_H}}{[X]^{n_H} + EC_{50}^{n_H}} \tag{2.1}$$

2.2 Measurement of I_{Ks}

The purpose of this section is to provide an outline of the protocols that were used to record I_{Ks}, and to describe some of the properties of the current under different recording conditions.

2.2.1 Recording of I_{Ks} in myocytes superfused with normal Tyrode's solution

The simultaneous activation of a number of membrane conductances that occurs when guinea pig ventricular myocytes are depolarized to plateau potentials makes it difficult to record individual currents, including I_{Ks}. However, it is possible to isolate I_{Ks}, and the procedures used are described below.

Myocytes were bathed in normal Tyrode's solution, held at −40 mV, and depolarized for 500 ms to test potentials between −40 and +70 mV. The depolarizations elicited inward $I_{Ca,\,L}$, and time-dependent outward I_K that increased with positive potential. The tail current amplitude was measured upon repolarization to −40 mV (Figure 1A). Under these conditions, the total outward tail current ($I_{K,\,tail}$) is comprised of both I_{Kr} (activated at potentials above −40 mV) and I_{Ks} (activated above 0 mV) (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a,b; Jones *et al.*, 2000). The $I_{K,\,tail}$–V relationship commonly has a distinctive biphasic shape (Figure 1B), where the ascending limb of the relationship at lower potentials is due to activation of I_{Kr} which saturates at potentials between +10 and +20 mV, and the additional increase of the tail amplitude at higher positive potentials is due to activation of I_{Ks} (Sanguinetti and Jurkiewicz, 1990; Liu and Antzelevitch, 1995; Heath

and Terrar, 1996a,b).

Effects of E4031

Application of the selective I_{Kr} blocker benzenesulfonamide E4031 (EC_{50} = 397 nM: Sanguinetti and Jurkiewicz, 1990) permits the isolation of I_{Ks} from total I_K in myocytes superfused with normal Tyrode's solution. The data in Figure 2A, B indicate that 3 μM E4031 strongly reduced the amplitude of tail currents elicited after depolarizations to potentials below +20 mV ($p < 0.001$; n = 7), but had no significant effect on the *increment* in tail current that occurred after depolarizations to more positive potentials.

2.2.2 Recording of I_{Ks} under K^+-, Ca^{2+}-free conditions

Superfusion of cells with K^+-, Ca^{2+}-free Tyrode's that contains 0.2–0.4 mM Cd^{2+} (i) suppresses K_o^+-dependent inwardly-rectifying I_{K1} and K_o^+-dependent, Cd^{2+}-sensitive I_{Kr}, (ii) facilitates the recording and measurement of I_{Ks} due to the increase in driving force for outward potassium current, and (iii) suppresses Cd^{2+}-sensitive Ca^{2+} currents (Sanguinetti and Jurkiewicz, 1990; Jones *et al.*, 1998). Under these conditions, prolonged (2 s) depolarizations from ≈ -30 mV to more positive voltages elicited time-dependent outward current, and repolarizations elicited outward tail currents (e.g., Figure 3A). In the majority of the experiments below, peak tail current amplitude (I_{tail}) was used to measure I_{Ks}.

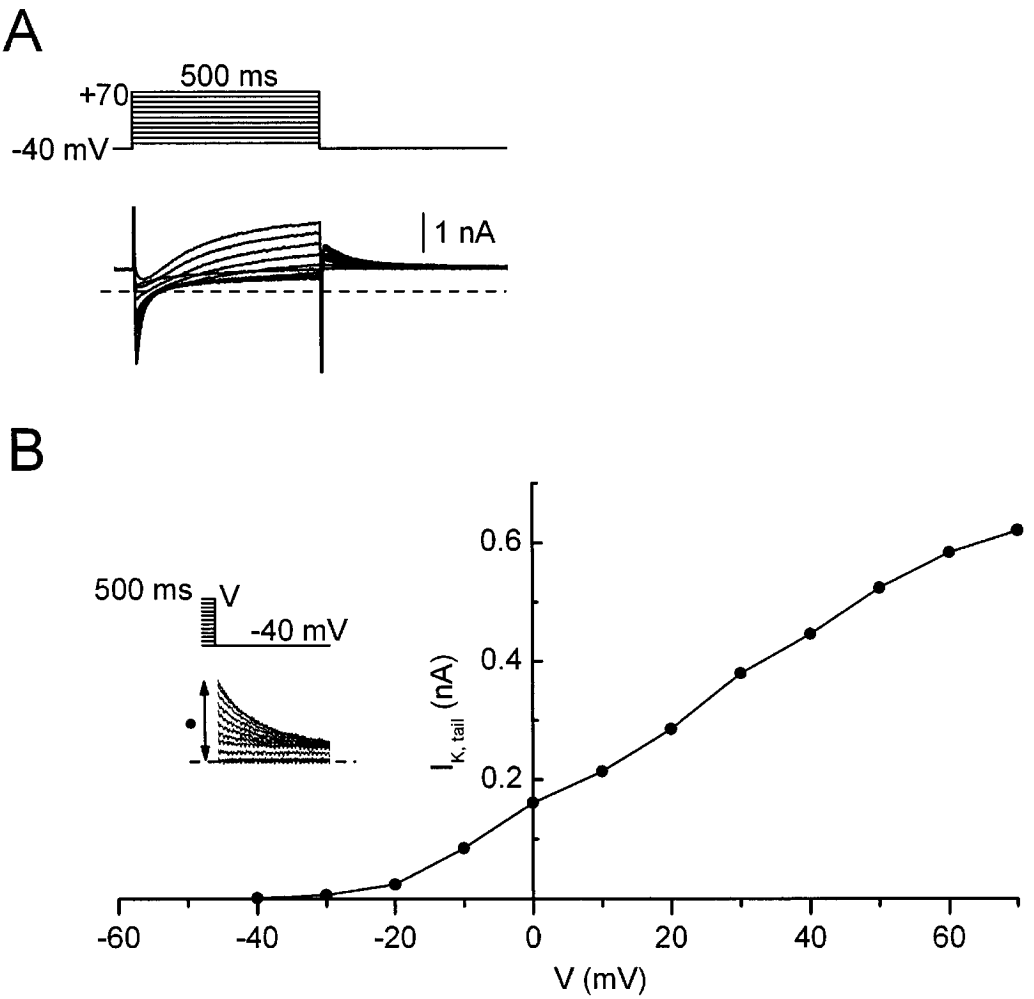

Figure 1: Whole-cell membrane currents recorded from a myocyte bathed in normal Tyrode's solution. (A) Membrane currents elicited by 500-ms depolarizations to test potentials between −40 mV and +70 mV. (B) The I–V relationship determined from measurements of the amplitude of tail currents obtained on repolarizations to −40 mV.

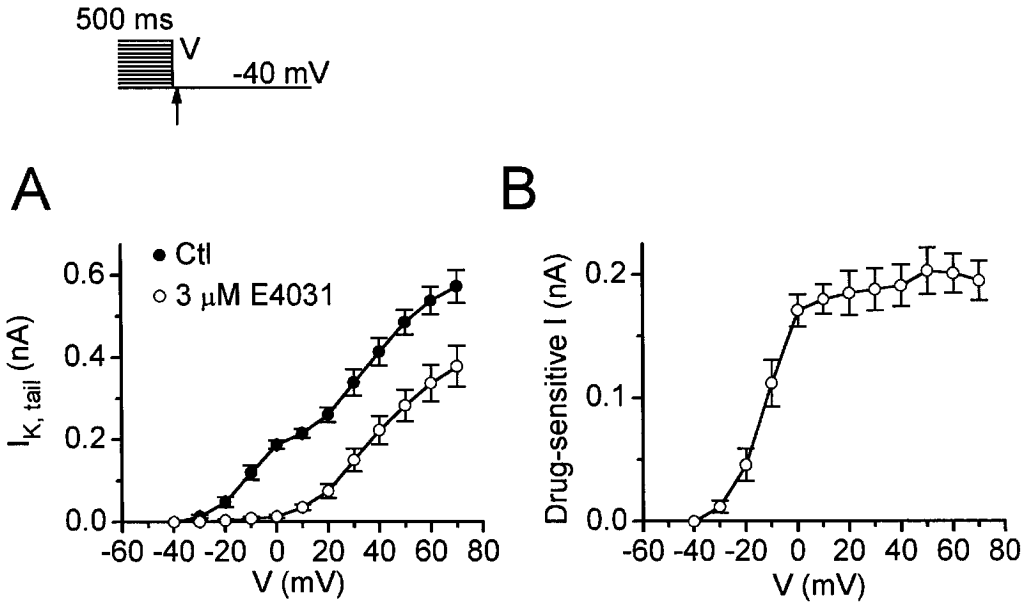

Figure 2: Effects of 3 μM E4031 on I_{K} tails recorded from myocytes bathed in normal Tyrode's solution. (A) Tail currents recorded upon repolarization to -40 mV after 500-ms depolarizing pulses to potentials between -40 and $+70$ mV. The $I_{\mathrm{K,\,tail}}$–V relationships were measured before and 5 min after addition of 3 μM E4031 (n = 7). (B) E4031-sensitive tail current obtained by subtraction of E4031 records from control records.

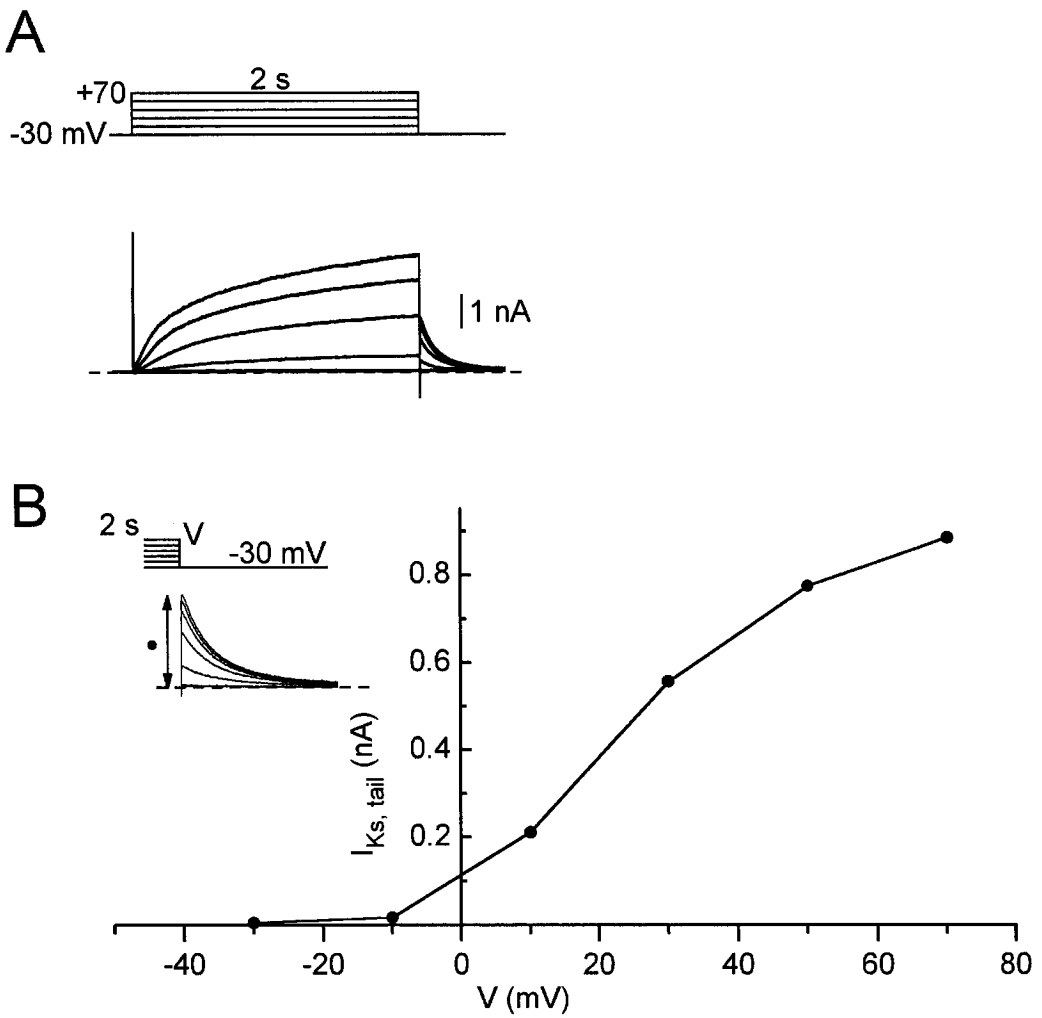

Figure 3: Recording of I_{Ks} under K^+-, Ca^{2+}-free conditions. (A) Records obtained from a representative myocyte that was depolarized with 2-s steps applied at 0.1 Hz. (B) The tail I–V relationship determined from the data in A. Inset: tail currents recorded in the same experiment.

Time- and voltage-dependent properties

Tail I–V relationships obtained by measuring the amplitudes of the tail currents on repolarization to −30 mV from more positive voltages (e.g., Figure 3B) have an activation threshold voltage near −10 mV and a single ascending slope, as expected of cardiac I_{Ks}. Slow, delayed activation without inactivation at positive potentials and deactivation of the current during repolarizations also closely resembled the profile of the slow component of cardiac delayed rectifier I_{Ks} (Sanguinetti and Jurkiewicz, 1990; Liu and Antzelevitch, 1995; Heath and Terrar, 1996a).

When required, tail I–V relationships were normalized (e.g., $I_{\text{tail (+90 mV)}} = 1.0$) and fitted with a Boltzmann function (equation 2.2) that has a $V_{0.5}$ (potential of half-maximal tail current amplitude) of +17 mV and a slope factor (S) of +15 mV:

$$I/I_{max} = 1/\{1 + e^{(V_{0.5}-V)/S}\} \tag{2.2}$$

The deactivating tail currents recorded upon repolarizations to −30 mV after 2 s depolarizations from −30 mV to +50 mV could be fitted with single or double exponential functions. In the case of monoexponential fits, the time constant of tails recorded from nine representative myocytes was 165 ± 17 ms (n = 9), a value that is similar to the fast (τ_f) values of ≈130 ms obtained in canine ventricular myocytes (Liu and Antzelevitch, 1995), 257 ms in guinea pig ventricular myocytes (Heath and Terrar, 1996b), and ≈120 ms in porcine sino-atrial node cells (Ono *et al.*, 2000).

Lack of effect of E4031 on the tail current

The effects of I_{Kr} blocker E4031 (5 μM) were investigated to establish that the time-dependent K^+ currents recorded under K^+-, Ca^{2+}-free conditions were predominantly

comprised of I_{Ks} and were relatively free of contamination by rapidly-activating I_{Kr}. The blocker had no significant effect on the amplitude of tail currents elicited at −30 mV after 500-ms depolarizations (n = 7) (e.g., Figure 4A, B), indicating that no detectable I_{Kr} was present under these experimental conditions.

Sensitivity to block by chromanol 293B

The selective I_{Ks} blocker chromanol 293B (293B) was used to confirm that the $I_{K,\,tail}$ measured in K^+-, Ca^{2+}-free solution had pharmacological properties consistent with those of I_{Ks}. Application of 50 μM 293B caused a reversible 80% inhibition of tail current amplitude (e.g. Figure 5A, B), as expected for action of this compound on cardiac I_{Ks} (Busch *et al.*, 1996). Similar results were obtained in other myocytes (also see Results).

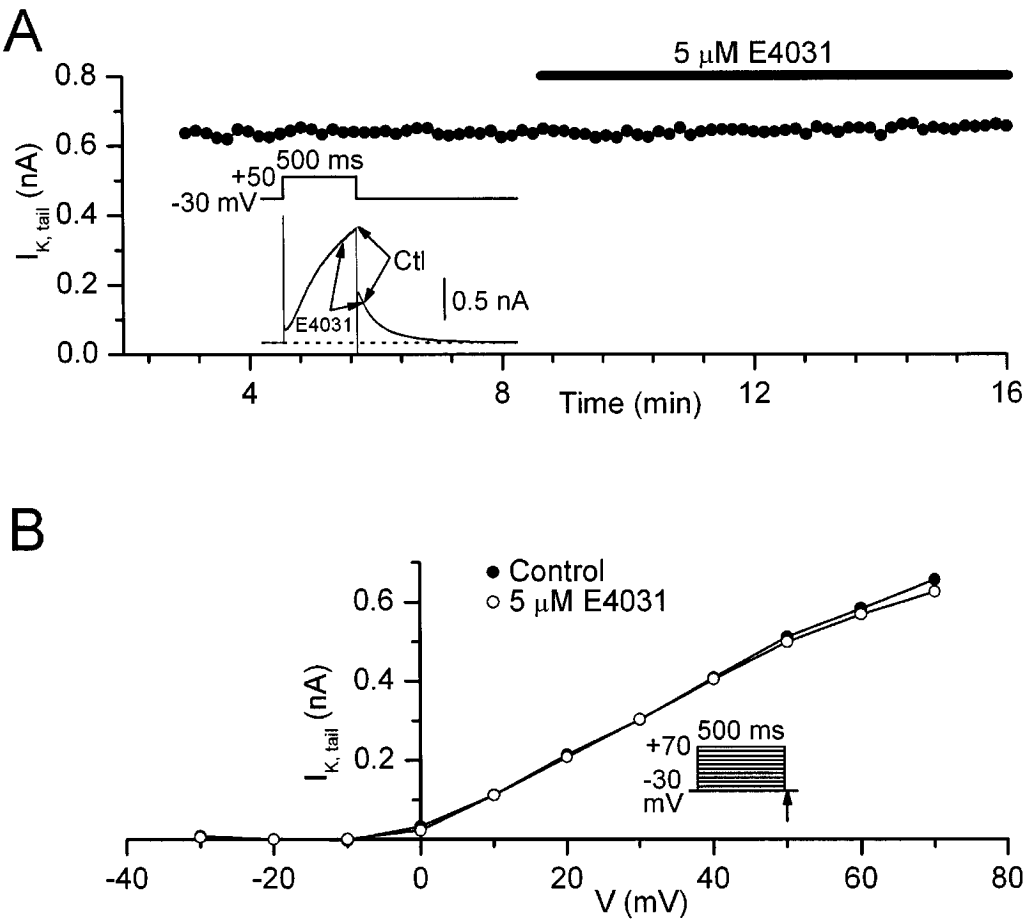

Figure 4: Effects of E4031 on $I_{K,\,tail}$ in myocytes superfused with K^+-, Ca^{2+}-free solution. (A) The time course of tail I_K amplitude before and during the application of 5 μM E4031. Inset: records (superimposed) obtained before (Ctl) and 6 min after addition of E4031. (B) Tail I–Vs obtained from a myocyte before (control) and 5 min after application of 5 μM E4031.

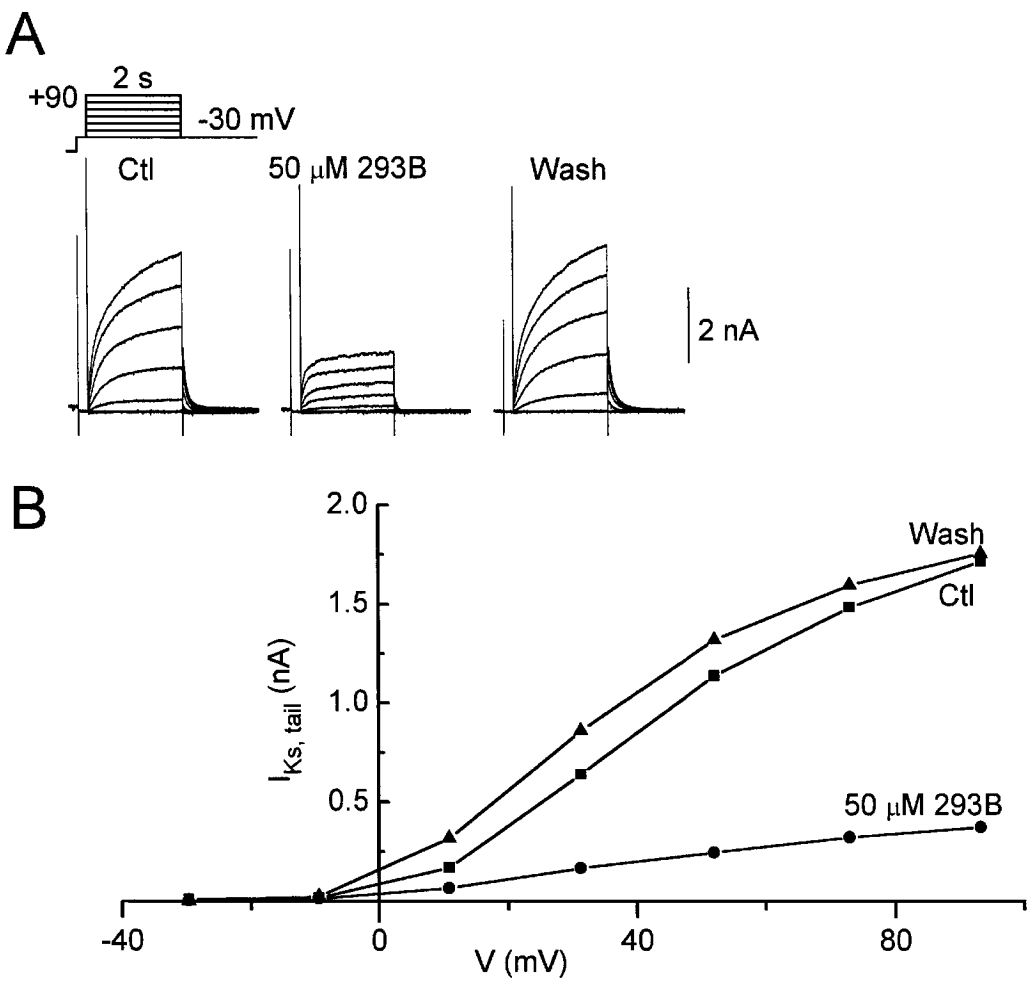

Figure 5: Inhibition of $I_{K,\,tail}$ by chromanol 293B in a representative myocyte superfused with K^+-, Ca^{2+}-free Tyrode's solution. (A) Records obtained at 5-min intervals. (B) Tail I–V relationships determined from the data in A.

3

Results

The first section of the Results (3.1) provides an overview of the effects of hyperosmotic and hyposmotic solutions on I_{Ks} and cell volume. Thereafter, the material is focused on I_{Ks} under isosmotic and hyposmotic conditions with regard to pharmacologic and voltage-dependent properties (section 3.2) and with regard to regulation of the current (sections 3.3 to 3.8). The last section (3.9) is concerned with correlatory experiments on I_{Ks}-like currents carried by K^+ channel subunits expressed in fibroblasts.

3.1 Effects of hyposmotic solution on I_{Ks} and myocyte volume

3.1.1 Stimulation of I_{Ks} hyposmotic swelling

To determine the effects of hyposmotic external solution on I_{Ks}, myocytes dialyzed with standard K^+ pipette solution were generally superfused with K^+-free 1T solution

and then with K^+-free hyposmotic solution. The osmolarity of the latter solution ranged from 0.9T to 0.5T, with the vast majority of the experiments being conducted with 0.75T and 0.5T solutions.

Figure 6 shows the data obtained from myocytes that were pulsed from −30 to +60 mV for 2 s every 30 s under 1T and hyposmotic 0.5T conditions. The superimposed records in Figure 6A illustrate that superfusion with 0.5T solution caused an approximate doubling of the amplitude of the I_{Ks} tail. The stimulation usually proceeded quite rapidly after an initial lag, and was readily reversed by re-admission of 1T solution (e.g., Figure 6B, top).

In some experiments, myocytes were bathed with 5.4-mM K^+, 5-μM E4031 solutions (instead of standard K^+-free solutions) to permit the recording of both I_{Ks} and inwardly-rectifying I_{K1}. In the example shown in Figure 6B, the amplitude of the I_{Ks} tail was monitored on repolarization to prepulse potential −30 mV, and the amplitude of outward-directed I_{K1} was monitored at a holding potential (−80 mV) that was just positive to E_K. As illustrated by the data in the figure, replacement of 1T solution by 0.5T solution caused both an increase in I_{Ks} (top panel) and a decrease in I_{K1} (bottom panel). The latter change in I_{K1} is consistent with a depolarizing shift in E_K as a consequence of rapid myocyte swelling.

The degree of swelling induced by superfusion with 0.5T solution was quite variable, ranging from 16 to 41% in twelve patched myocytes bathed with K^+-free solutions (Figure 6C). In these myocytes, the increase in the amplitude of the I_{Ks} tail ranged from 67 to 143%. A plot of the increase in I_{Ks} amplitude versus the increase in myocyte volume (Figure 6C) suggests that there may have been a correlation between the two variables under 0.5T conditions, but more experiments would be required to

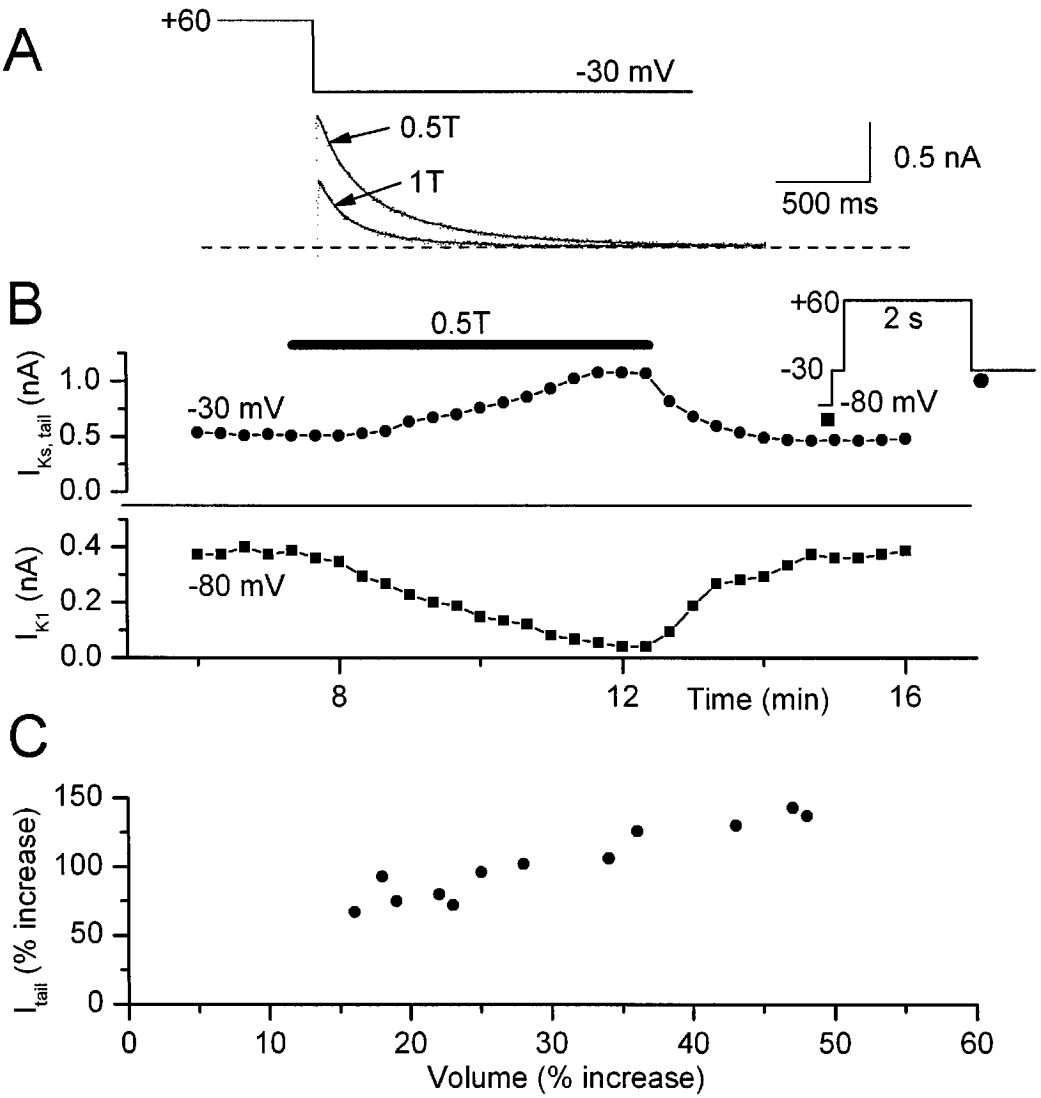

Figure 6: Effects of hyposmotic 0.5T solution on myocyte K^+ currents and myocyte volume. (A) I_{Ks} tails (−30 mV) after 2-s depolarizations to +60 mV in a representative myocyte before (1T) and 5 min after admission of 0.5T bathing solution. K^+-free solutions. The dashed line indicates the zero-current level. (B) Time course of changes in the amplitudes of the I_{Ks} tail at −30 mV (top) and outward I_{K1} at holding potential −80 mV (bottom) in a myocyte exposed to 0.5T solution. The 1T and 0.5T solutions contained 5.4 mM K^+ and 5 μM E4031. (C) Percentage increases (reference 1T) in the amplitude of tail I_{Ks} and myocyte volume after exposure of twelve myocytes to 0.5T solution for 4–7 min. K^+-free solutions.

establish this point with certainty.

3.1.2 I_{Ks}–external osmolarity relationship

Figure 7A summarizes the I_{Ks} data obtained from a large number of myocytes that were superfused with 1T solution and then exposed to a test anisosmotic solution. In each case, the amplitude of the test I_{Ks} tail was expressed as a fraction of the control (1T) amplitude, and a plot of these fractions versus the inverse of test osmolarity is shown in Figure 7. The total number of satisfactory experiments was 170, and the data from these are well described by the dose–response function $y = a1 + (a2 - a1)/(1 + 10^{(\log x_0 - x)p})$, where $a1$ and $a2$ are asymptotes, $\log x_0$ is centre, and p is a Hill slope. Since the relative current amplitude at 1/relative osmolarity was defined to be 1.0, $\log x_0$ was by definition 1.0, $a1$ was set to 0, $a2$ was set to 2.0, and p was estimated to be 1.87 $\pm$ 0.17 from the curve fit (Figure 7).

3.1.3 Stimulation by hyperosmotic internal solution

A second protocol used to swell myocytes was to bathe them in 1T solution and dialyze them with hyperosmotic 1.5T or 2.2T pipette solution instead of standard 1T solution. With this protocol, the amplitude of I_{Ks} increased rapidly within a very short time after patch breakthrough, and this increase appeared to be coincident with an increase in cell volume. Since it was difficult to make an accurate determination of the steady-state amplitude of control (pre-swelling) I_{Ks} for evaluation of swelling-induced stimulation, the extent of the stimulation was evaluated from the change in I_{Ks} that occurred when swollen cells were deflated to initial volume by attenuation of the osmotic gradient (replacement of 1T superfusate by hyperosmotic superfusate).

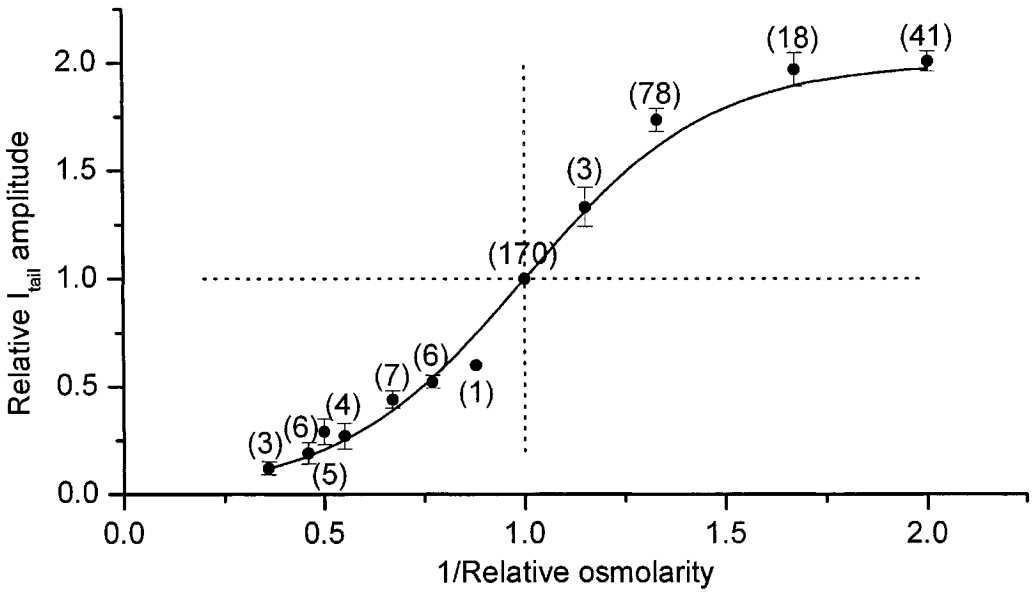

Figure 7: Dependence of I_{Ks} tail amplitude on the osmolarity of the external solution. Current amplitudes under anisosmotic test conditions were expressed as fractions of 1T (control) amplitudes and plotted against the inverse of test osmolarity. The dose–response fit to the data (see text for details) has a Hill slope of 1.87 ± 0.17. Numbers of myocytes in parentheses.

In four myocytes patched with 1.5T pipette solution, the amplitude of stimulated I_{Ks} was 75 ± 5% larger than that of control I_{Ks} ($p < 0.001$); in sixteen myocytes patched with 2.2T solution, the amplitude of stimulated I_{Ks} was 121 ± 11% larger ($p < 0.001$). These results suggest that osmotic gradient and resultant cell swelling, rather than absolute osmolarity, are related to hyposmotic stimulation of I_{Ks}.

3.1.4 Osmosensitive I_{Ks} and the ventricular action potential

Computer simulations were preformed using Cell Electrophysiology Simulation Environment (CESE) v. 1.3.3 (Missan and McDonald, 2005) and the Luo–Rudy phase II model of cardiac electrical activity (Luo and Rudy, 1994a,b; Faber and Rudy, 2000) to assess the changes in the configuration and behaviour of the ventricular action potential likely to be caused by the modulation of I_{Ks} by anisosmotic conditions. The maximal conductance of I_{Ks} in the model was adjusted in accord with the experimental data provided in Figure 7. As shown in Figure 8A, the duration of the basal (1T) simulated action potential at the 90% repolarization level (APD_{90}) was 160 ms. When I_{Ks} amplitude was increased to simulate the effects of myocyte superfusion with 0.75T and 0.5T solutions, APD_{90} decreased to 136 and 131 ms, respectively. Conversely, when I_{Ks} was decreased in accord with hyperosmotic 1.5T data, the APD_{90} increased to 210 ms.

As an initial step in determining the possible consequences of modulated action potential duration on cardiac electrical activity, the effects of stimulation frequency on myocyte repolarization (i.e., restitution) were investigated. The model myocyte was paced with a basic cycle length of 800 ms (interval S1) for 10 stimulations. After the basal stimulation train, the model was stimulated at a variable cycle length (S2)

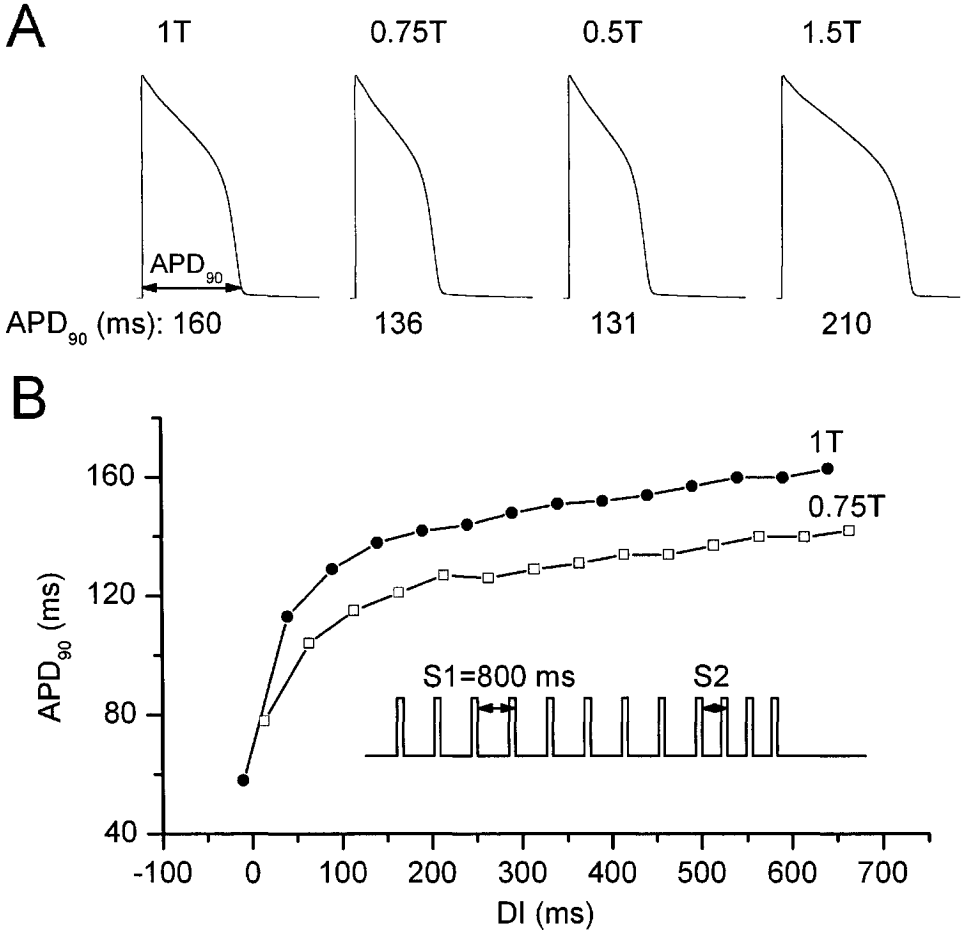

Figure 8: Computer simulations of the effects of changes in I_{Ks} amplitude on the action potential duration and restitution curve. (A) Simulated action potentials. The maximal conductance of I_{Ks} in the Luo–Rudy phase II model was modified to simulate the effects of myocyte superfusion with 1T, 0.75T, 0.5T, and 1.5T solutions. (B) Simulated restitution curves for control (1T) and 0.75T conditions. The model was paced with a basic cycle length of 800 ms (S1) for 10 stimulations, and then with a variable cycle length (S2) for 3 stimulations. The action potential duration at the 90% repolarization level (APD_{90}) was measured and plotted against the DI length.

for 3 stimulations, and the APD_{90} was measured and plotted against the S2 interval. The diastolic interval (DI) between the last control action potential (at S1 basic cycle length) and first test action potential (at S2 basic cycle length) has been calculated as $DI = S2 - APD_{90(S1)}$. The DI–APD_{90} relationship is depicted in Figure 8B; the APD_{90} was smaller under 0.75T conditions over a wide range of DI intervals (10–700 ms). Moreover, the slope of the restitution curve at high pacing rates (S2 $<$ 200 ms) was much steeper under 1T conditions than under 0.75T conditions.

3.2 Properties of I_{Ks} under 1T and 0.75T conditions

3.2.1 Effects of K^+ channel blockers

A limited pharmacological profile of hyposmotic-stimulated I_{Ks} was obtained by investigating the response of the current to application of three drugs that are known to inhibit cardiac K^+ currents. The three drugs were the $I_{K,\,ATP}$ blocker glibenclamide (Findlay, 1992), the I_{Kr} blocker E4031 (Heath and Terrar, 1996a; Jones *et al.*, 1998), and the I_{Ks} blocker chromanol 293B (293B) (Busch *et al.*, 1996; Fujisawa *et al.*, 2000). Myocytes depolarized to +50 mV for 500 ms at 0.1 Hz were superfused with K^+-free 1T solution and then with K^+-free 0.75T hyposmotic solution, and the blockers were added either during the 1T or the 0.75T superfusion. Glibenclamide (1–3 μM) had no significant effect on the amplitude of the I_{Ks} tail during either 1T superfusion (increase of 5 $\pm$ 3%; n = 5) or 0.75T superfusion (increase of 3 $\pm$ 3%; n = 4). Similarly, 5–10 μM E4031 had no significant effect under either the 1T (decrease of 1 $\pm$ 4%; n = 5) or the 0.75T (decrease of 3 $\pm$ 4%; n = 11) conditions.

In contrast to the ineffectiveness of glibenclamide and E4031, 293B had strong

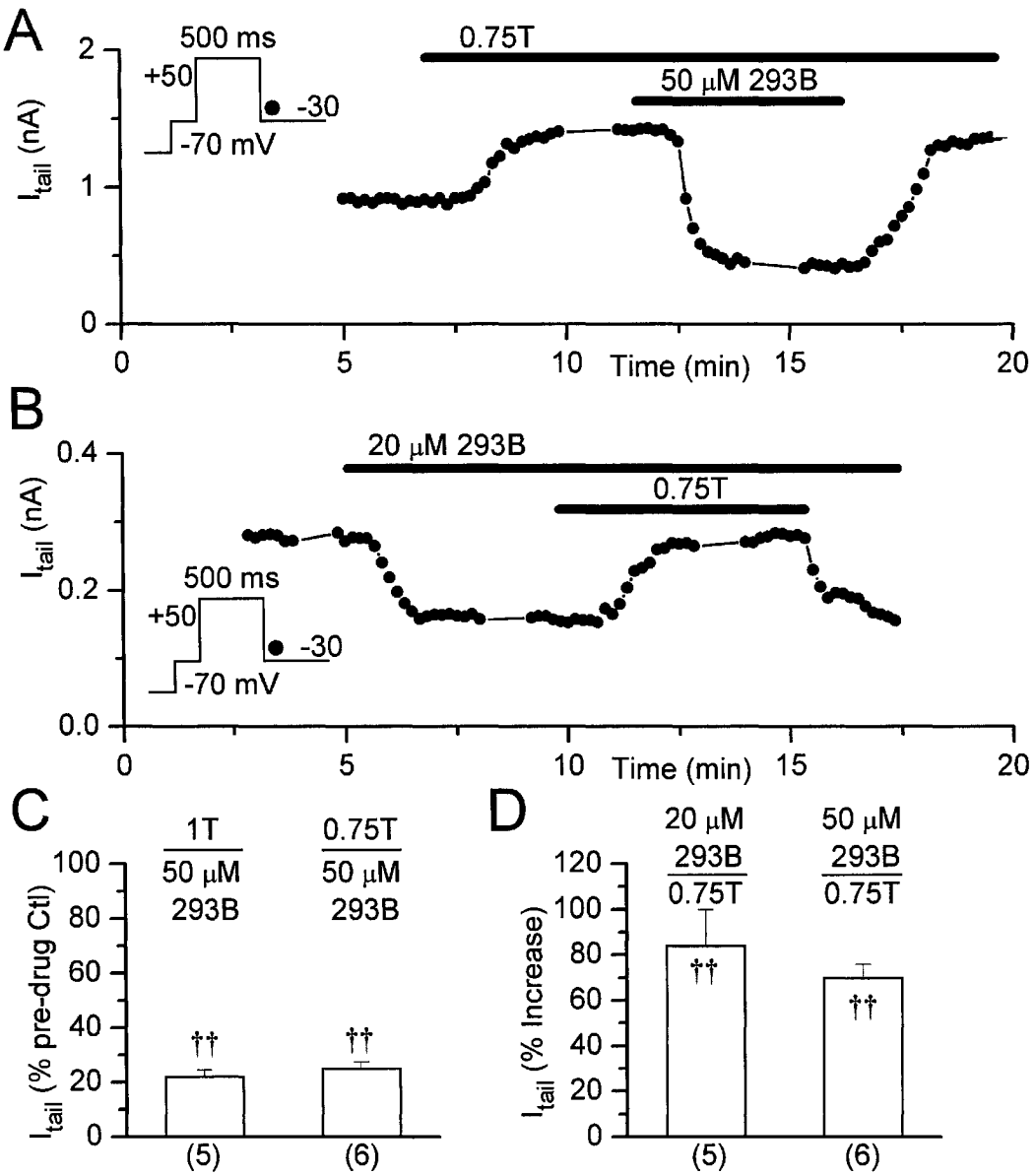

Figure 9: Effects of 293B on I_{Ks} in myocytes superfused with 1T and 0.75T solution. (A) Reversible inhibition of tail I_{Ks} by 50 μM 293B in a myocyte exposed to 0.75T solution. (B) Stimulation of I_{Ks} by 0.75T solution following partial inhibition of the current by 20 μM 293B. (C) Effects of 5-min treatments with 50 μM 293B on I_{Ks} in myocytes bathed in 1T and 0.75T solutions. (D) Stimulation of 293B-inhibited I_{Ks} by hyposmotic solution. †† $p < 0.001$, paired t-test versus pre-drug value. Numbers of myocytes in parentheses.

inhibitory effects. At a 50 μM concentration, the drug reduced the amplitude of the I_{Ks} tail by 78 $\pm$ 3% (n = 5; $p < 0.001$) and 75 $\pm$ 3% (n = 6; $p < 0.001$) under 1T and 0.75T conditions, respectively (Figure 9A, C). In those myocytes that were tested during superfusion with 0.75T solution, washout of the drug restored the current to 95 $\pm$ 5% of its pre-drug amplitude (e.g., Figure 9A).

To investigate whether partial block of I_{Ks} by 293B modified the response of the current to hyposmotic solution, myocytes were pretreated with 20 or 50 μM 293B for 5 min and then exposed to hyposmotic 293B solution. In the example shown in Figure 9B, 20 μM 293B inhibited the I_{Ks} tail by $\approx$45%, and subsequent exposure to hyposmotic solution stimulated the (inhibited) current by $\approx$80%. Overall, 20 μM 293B inhibited the current by 46 $\pm$ 7% ($p < 0.01$), and hyposmotic solution stimulated the (inhibited) current by 84 $\pm$ 16% (n = 5; $p < 0.001$); the corresponding results with 50 μM 293B were an inhibition of 76 $\pm$ 4% ($p < 0.001$) and a stimulation of 70 $\pm$ 6% (n = 6; $p < 0.001$) (Figure 9D). In summary, substantial tonic block of the basal I_{Ks} had no effect on hyposmotic stimulation of the current (control 73 $\pm$ 5%, n = 78).

3.2.2 Voltage dependence

To evaluate the voltage dependencies of basal and hyposmotic-stimulated I_{Ks}, isochronic (2-s) tail I–V relationships were determined from experimental data recorded from eight myocytes. In each myocyte, I–V runs were performed under control (1T) conditions, and again 5 min after admission of 0.6T solution (Figure 10A). Each I–V relationship was normalized by assigning a value of 1.0 to the amplitude of the tail current that was elicited after the depolarization to +90 mV, and the resultant mean

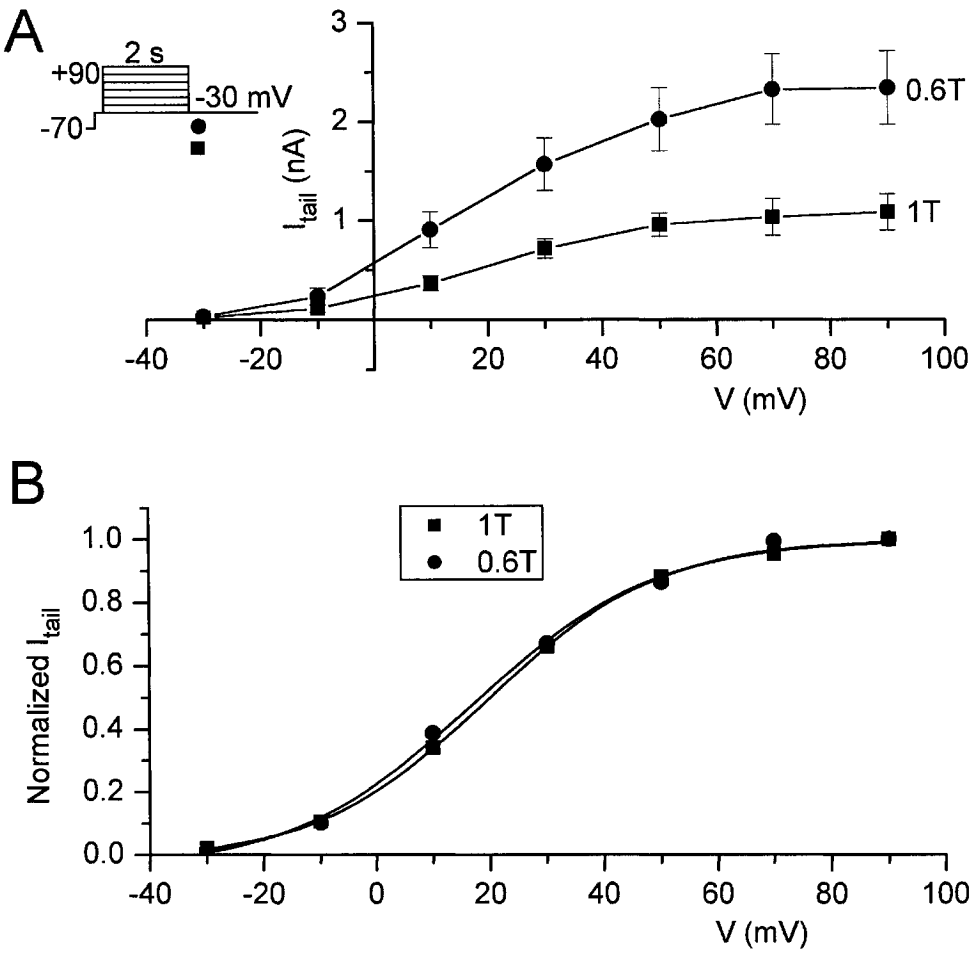

Figure 10: Current–voltage and isochronic activation relationships for basal and 0.6T-stimulated I_{Ks}. (A) Tail I–V relationships for I_{Ks} (1T) and I_{Ks} (0.6T) determined using 2-s test pulses from −30 to +90 mV at 0.1 Hz. The data are from eight myocytes. (B) Boltzmann fits to normalized mean values taken from the tail current–voltage relationships in A.

data from 1T and 0.6T sets were each fitted with a Boltzmann function (see equation 2.2). As indicated by the "activation" curves in Figure 10B, both the half-maximal voltage ($V_{0.5}$) (+17 ± 1.8 mV) and the slope factor (S) (+16 ± 1.3 mV) that describe the 0.6T data are similar to those that describe the 1T data (+19 ± 0.6 mV; +15 ± 0.4 mV). This result suggests that hyposmotic swelling has little effect on the activation of I_{Ks}. However, it is important to note that the "activation" relationships obtained from these experiments only approximate the true relationships because I_{Ks} continues to activate over times >2 s (Sanguinetti and Jurkiewicz, 1990; Liu and Antzelevitch, 1995; Heath and Terrar, 1996a).

3.2.3 Time courses of activation and deactivation

The kinetics of deactivation of I_{Ks} under 1T and 0.75T conditions were compared by fitting double exponentials to tail currents elicited on repolarization to −30 mV after 2-s pulses to +50 mV. The measurements were taken 4 times for each myocyte: under 1T conditions shortly before the application of 0.75T solution, after I_{Ks} reached steady-state under 0.75T conditions, shortly before re-application of 1T solution, and at steady state thereafter. This protocol was suitable for the assessment of possible quick changes in the time course of deactivation in response to changes in external osmolarity. The slow time constant (overall mean 363 ± 23 ms, n = 36) has increased by 16 ± 26 ms upon application of 0.75T solution and decreased by 40 ± 32 ms following its removal (n = 9); the fast time constant (overall mean 97 ± 5 ms, n = 36) has increased by 6 ± 5 ms after application of 0.75T solution and decreased by 9 ± 7 ms following its removal (n = 9). None of the changes were significant from 0 ms change in the time constant (unpaired t-test).

The noticeable slow increase in the slow and fast time constants of deactivation has been detected during the course of several experiments; in some cases, the slow time constant of deactivation increased by more than 100 ms over 20–30 min of observation. However, this slow increase in the time constants of deactivation does not correlate with changes in the extracellular osmolarity and therefore it is unlikely that 0.75T solution changes the I_{Ks} deactivation kinetics.

I_{Ks} traces from 2-s depolarizations to +50 mV were fitted with double exponentials for estimation of activation kinetics. The measurements were taken under 1T conditions, during application of 0.75T solution, and following the return to 1T solution. There was no significant change in either the fast time constant (average values of 122 ± 22, 131 ± 36, and 128 ± 55 ms) or the slow time constant (1125 ± 155, 1238 ± 130, and 1395 ± 140 ms) under the three conditions (n = 6 myocytes).

In summary, it appears that neither activation nor deactivation kinetics of I_{Ks} were affected by changes (1T, 0.75T, 1T) in external osmolarity.

3.3 Regulation of I_{Ks} by PKA and PKC

It is well established that PKA and PKC have positive regulatory influences on I_{Ks} in guinea pig ventricular myocytes. For example, the amplitude of basal I_{Ks} is markedly increased when the adenylate cyclase–cAMP–PKA pathway is stimulated by application of FSK or ISO (Walsh and Kass, 1988; Yazawa and Kameyama, 1990; An *et al.*, 1999), and is also increased when PKC is activated by application of active phorbol ester (Tohse *et al.*, 1987; Walsh and Kass, 1988; Tohse *et al.*, 1990; Asai *et al.*, 1996). Clearly, the stimulation of I_{Ks} by hyposmotic swelling could be mediated by activation of one or both of these kinases. To investigate this possibility, I_{Ks} was studied

under conditions designed to perturb the activities of PKA and/or PKC, with two questions in mind: (i) does inhibition of PKA (PKC) modify the response of I_{Ks} to hyposmotic stress and (ii) does pre-stimulation of I_{Ks} via the PKA (PKC) pathway occlude further stimulation via hyposmotic swelling?

3.3.1 Regulation by PKA

Acetylcholine (ACh) and two isoquinolinesulfonamide inhibitors of cyclic-nucleotide -dependent kinases, H8 (Hidaka and Kobayashi, 1992) and H89 (Chijiwa *et al.*, 1990; Davies *et al.*, 2000), were used to inhibit PKA activity, and isoproterenol (ISO) and forskolin (FSK) were used to enhance kinase activity.

Experiments with ACh

ACh antagonizes PKA-mediated stimulation of cardiac ionic currents by causing an activation of receptor-coupled G_i with consequent downregulation of adenylate cyclase activation (Harvey and Hume, 1989; Shuba *et al.*, 1990; McDonald *et al.*, 1994). However, ACh had only minor effects on I_{Ks}. A 10-μM concentration increased the amplitude of the current by 10 $\pm$ 2% (n = 8; $p < 0.01$) under 1T conditions, but neither 3 μM (n = 3), 10 μM (n = 1), nor 30 μM ACh (n = 3) had a significant effect under 0.75T conditions.

Experiments with H8

In this series of experiments, myocytes were dialyzed with pipette solution that contained 200 μM H8 (EC_{50} (PKA) $\approx$1.2 μM: Hidaka and Kobayashi, 1992), and superfused with either 0 mM K^+ or 4.6-mM K^+ solution. Whether by direct or indirect

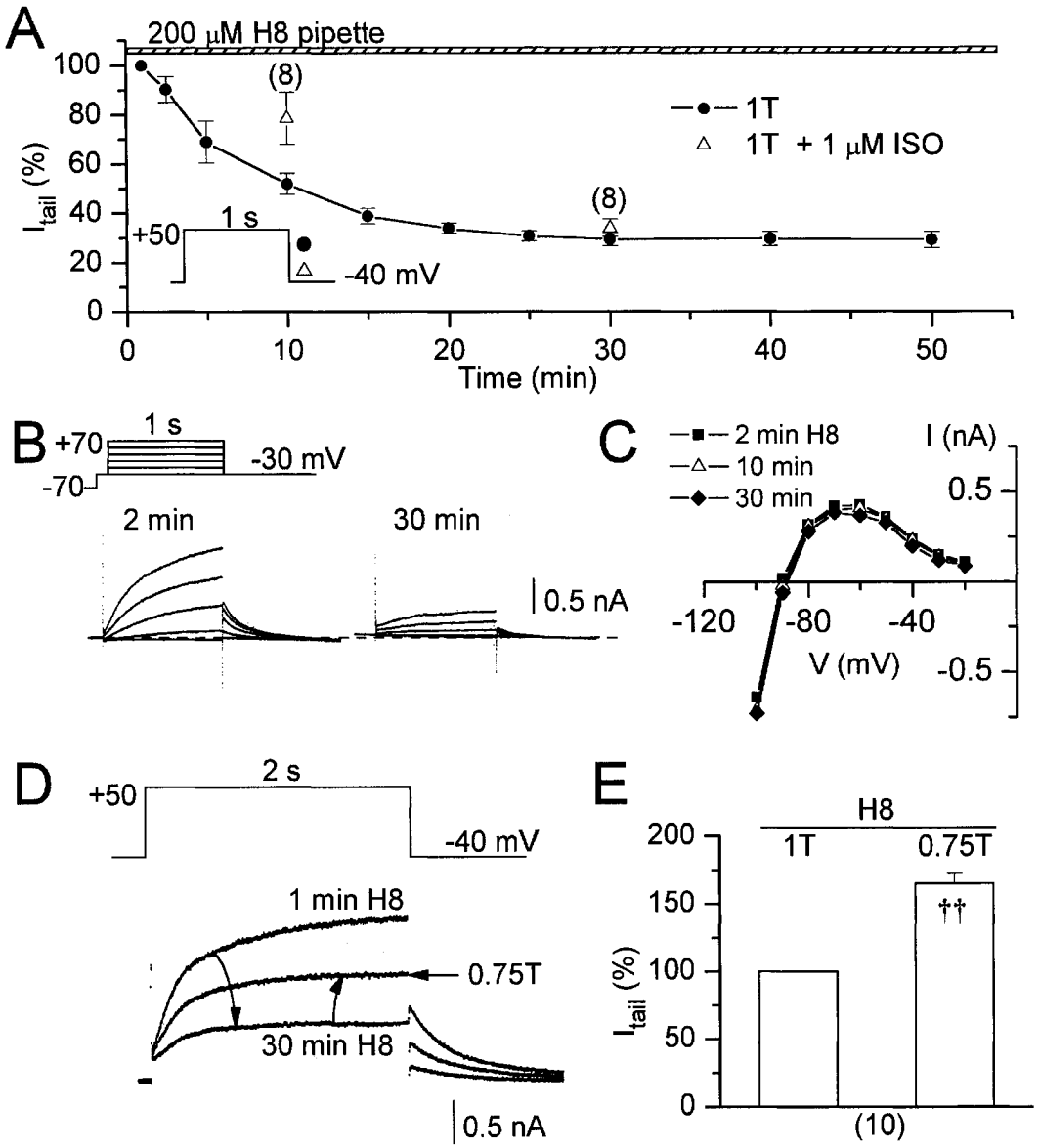

Figure 11: Effects of H8 on K^+ currents. Myocytes were dialyzed with 200-μM H8 pipette solution, and superfused with either 0-mM or 4.6 mM K^+ solution that contained 0.4 mM Cd^{2+}. (A) Inhibition of I_{Ks} by H8 dialysate. Tail current amplitudes were referenced to those recorded at 1 min post-patch (100%). Filled circles: data from "control" H8-dialyzed myocytes (nineteen up to 30 min, seven up to 50 min). Open triangles: data from test myocytes treated with 1 μM ISO at either 8 or 28 min post-patch. (B) Records of I_{Ks} obtained from an H8-dialyzed myocyte. (C) Lack of effect of H8 on the I_{K1}–V relationship. (D) Stimulation of I_{Ks} by 0.75T solution in a myocyte pretreated for 30 min with H8 dialysate. (E) Summary of the results obtained in experiments like D. †† $p < 0.001$, paired t-test. Number of myocytes in parentheses.

action of the inhibitor, dialysis with H8 solution had a marked, time-dependent inhibitory effect on the amplitude of basal I_{Ks}. Compared to the amplitude measured at 1 min post-patch, the amplitudes at 10 and $\geq$20 min post-patch were attenuated by $48 \pm 7\%$ (n = 19; $p < 0.001$) and $\approx$70%, respectively (Figure 11A, B). This inhibition was not due to a general deterioration of the myocytes because metabolically-sensitive inwardly-rectifying I_{K1} was little affected by the H8 treatment (e.g., Figure 11C).

To determine whether the H8 treatment caused effective inhibition of PKA, test myocytes were challenged with a single application of a relatively high (1 μM) concentration of ISO. The β-agonist had a strong stimulatory effect on I_{Ks} when it was applied at $\approx$8 min post-patch, but a relatively weak stimulatory effect when applied at $\approx$28 min post-patch (Figure 11A, open triangles). This result suggests that prolonged treatment with H8 was an effective means of inhibiting PKA activity.

To determine whether inhibition of PKA affected the stimulation of I_{Ks} by hyposmotic solution, myocytes were pretreated with H8 for $\approx$30 min, and then exposed to 0.75T solution. In eight such trials, exposure to hyposmotic solution increased the amplitude of the current by $83 \pm 14\%$ ($p < 0.001$) (Figure 11B–E).

Experiments with H89

Similarly to H8, H89 inhibits PKA by interfering at the level of the catalytic domain of the kinase (EC_{50} $\approx$50 nM: Hidaka and Kobayashi, 1992). In previous studies on heart cells, H89 has been used at 1 μM (e.g., Yuan and Bers, 1995; Zhang *et al.*, 2003; Jurevicius *et al.*, 2003; duBell and Rogers, 2004) and 10 μM (Yuan and Bers, 1995; Shuba and McDonald, 1997; Lei *et al.*, 2000) to investigate the role of PKA in the

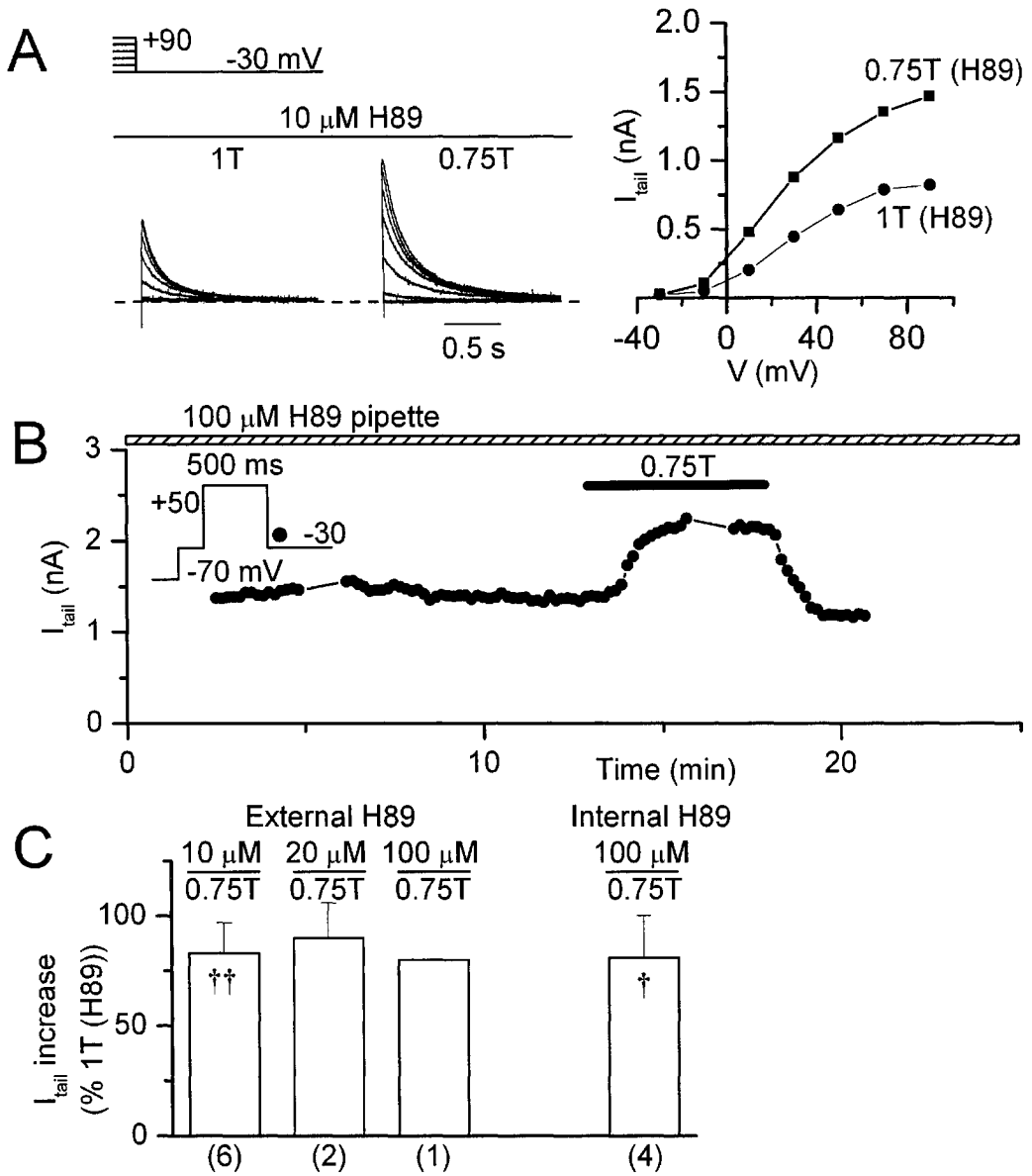

Figure 12: Lack of effect of H89 on the stimulation of I_{Ks} by hyposmotic 0.75T solution. (A) Data obtained from a myocyte pretreated with 10 μM H89 for 16 min, and then exposed to 0.75T drug solution for 6 min. Left: records of tail I_{Ks} elicited after 2-s depolarizations to more positive potentials; right: tail I–V relationships. (B) Time course of I_{Ks} amplitude in a myocyte dialyzed with 100-μM H89 and exposed to 0.75T solution. (C) Summary of the results obtained using external (left) and internal (right) H89. † $p < 0.01$, †† $p < 0.001$, paired t-tests versus pre-0.75T amplitude. Numbers of myocytes in parentheses.

regulation of ion channels. In the present study, myocytes were pretreated with 10–100 μM H89, and then exposed to 0.75T H89 solution. As illustrated by the data in Figure 12A, the hyposmotic solution increased I_{Ks} by 83 $\pm$ 13% (n = 6) in myocytes pretreated with 10 μM H89 for 14 $\pm$ 2 min. Similar-sized percentage increases were observed in two myocytes pretreated with 20 μM H89 for 21 min, and in one myocyte pretreated with 100 μM H89 for 20 min (Figure 12C).

A second method used to block PKA-mediated actions on ion channels has been to apply 1–20 μM H89 via the patch pipette (Groh *et al.*, 1996; Lien *et al.*, 2002; Mei *et al.*, 2004). In the present study, dialysis with 100-μM H89 solution for $\approx$16 min had little effect on basal I_{Ks}, and no apparent effect on the degree of stimulation of I_{Ks} by 0.75T solution (increase of 80 $\pm$ 17%; n = 4; $p < 0.05$) (Figure 12B, C).

Effect of FSK pretreatment on hyposmotic stimulation of I_{Ks}

To determine whether strong stimulation of I_{Ks} via the PKA pathway occludes additional stimulation by hyposmotic swelling, myocytes were treated with 1 or 5 μM FSK for 5–8 min prior to superfusion with hyposmotic 0.75T solution. Figure 13A shows families of current traces obtained during one of the trials with 5 μM FSK. Under 1T conditions, the diterpene markedly increased the amplitude of I_{Ks} (and also activated CFTR Cl^- channels as indicated by the shifts in time-independent current levels). Subsequent exposure of the myocyte to hyposmotic FSK solution caused an additional strong stimulation of I_{Ks} that was reversed after re-admission of isosmotic FSK solution. In six experiments with 5 μM FSK, and ten with 1 μM FSK, exposure to hyposmotic FSK solution increased the amplitude of the I_{Ks} tail by 77 $\pm$ 5% ($p < 0.001$) and 65 $\pm$ 7% ($p < 0.001$), respectively (Figure 13B, C).

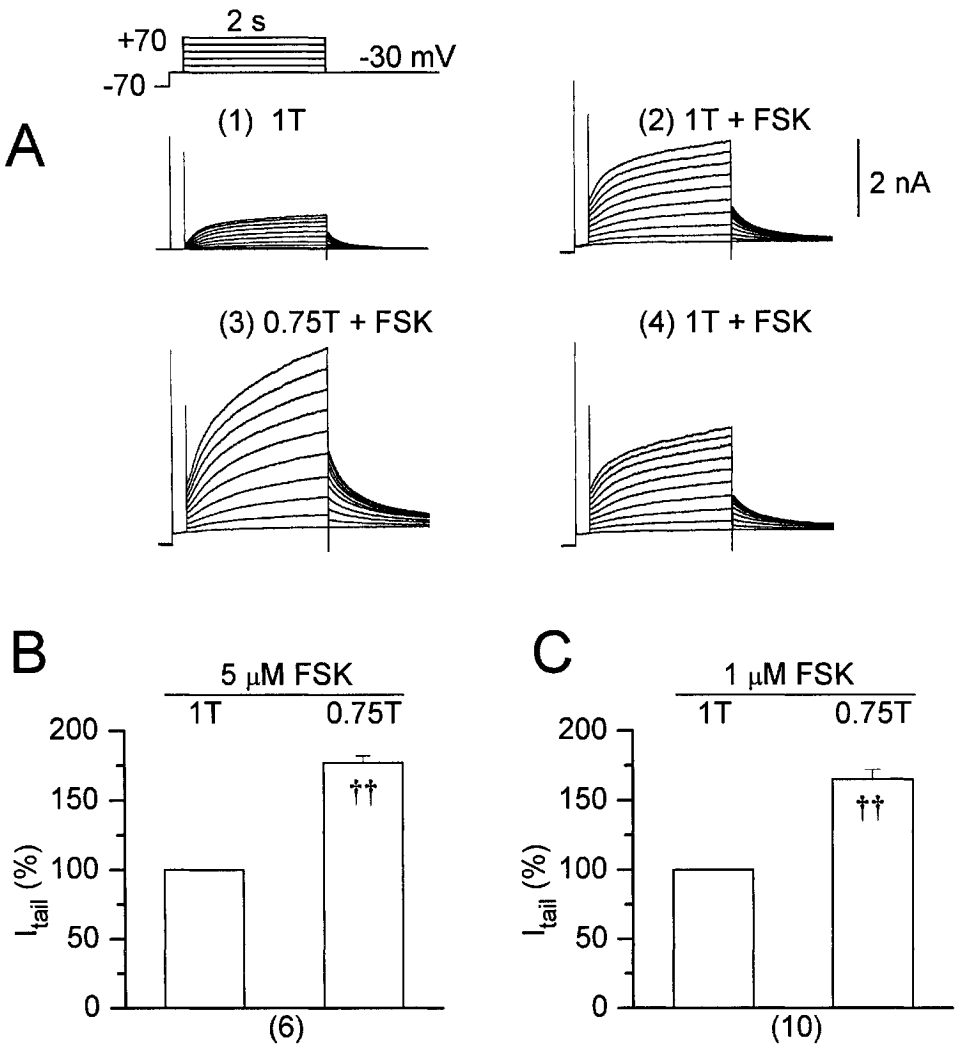

Figure 13: Additive stimulation of I_{Ks} by FSK and hyposmotic solution. (A) Families of current traces obtained from a myocyte that was pretreated with 5 μM FSK under 1T conditions, and then exposed to 0.75T + FSK solution. The elapsed time between collection of each set of traces was approximately 6 min. (B, C) Summary of results obtained from myocytes treated with 5 and 1 μM FSK. The amplitudes of I_{Ks} tails were measured on repolarizations to -30 mV after 500-ms depolarizations to $+50$ mV just before (FSK(1T), 100%) and $\approx$6 min after superfusion with 0.75T solution (FSK(0.75T)). †† $p < 0.001$, paired t-test. Numbers of myocytes in parentheses.

3.3.2 Regulation by PKC

Investigation of the regulation of I_{Ks} by PKC was carried out in much the same way as described above for regulation by PKA. In brief, PKC was inhibited by using either H7 (Nishikawa *et al.*, 1984) or bisindolylmaleimide I (Bis) (Toullec *et al.*, 1991), and PKC was activated by using phorbol 12-myristate 13-acetate (PMA) (e.g., Castagna *et al.*, 1982).

Experiments with H7

In functional studies on cells and tissues, H7 (EC_{50} (PKC) $\approx$6 μM: Hidaka and Kobayashi, 1992) is usually added to the external solution at concentrations of 10–50 μM to suppress the activity of PKC (e.g., Hagiwara *et al.*, 1992; Forstner *et al.*, 1994; Duan *et al.*, 1995; Chu *et al.*, 2003). For example, Tohse *et al.* (1987, 1990, 1992) and Matsubayashi *et al.* (1999) used a 10-μM concentration to evaluate the regulation of I_{Ks} by PKC in guinea pig ventricular myocytes.

In the present study, myocytes were dialyzed with 50-μM H7 pipette solution, pretreated with external 50 μM H7 for 10 min, and then exposed to hyposmotic H7 solution. As illustrated by the data in Figure 14A, the inhibitor caused a rapid and then a slower reduction in the amplitude of the I_{Ks} tail during the pretreatment period in 1T solution; however, the subsequent exposure to hyposmotic H7 solution resulted in a marked stimulation of the current. In eight experiments of this type, the hyposmotic solution increased the amplitude of I_{Ks} by 89 $\pm$ 11% (Figure 14C).

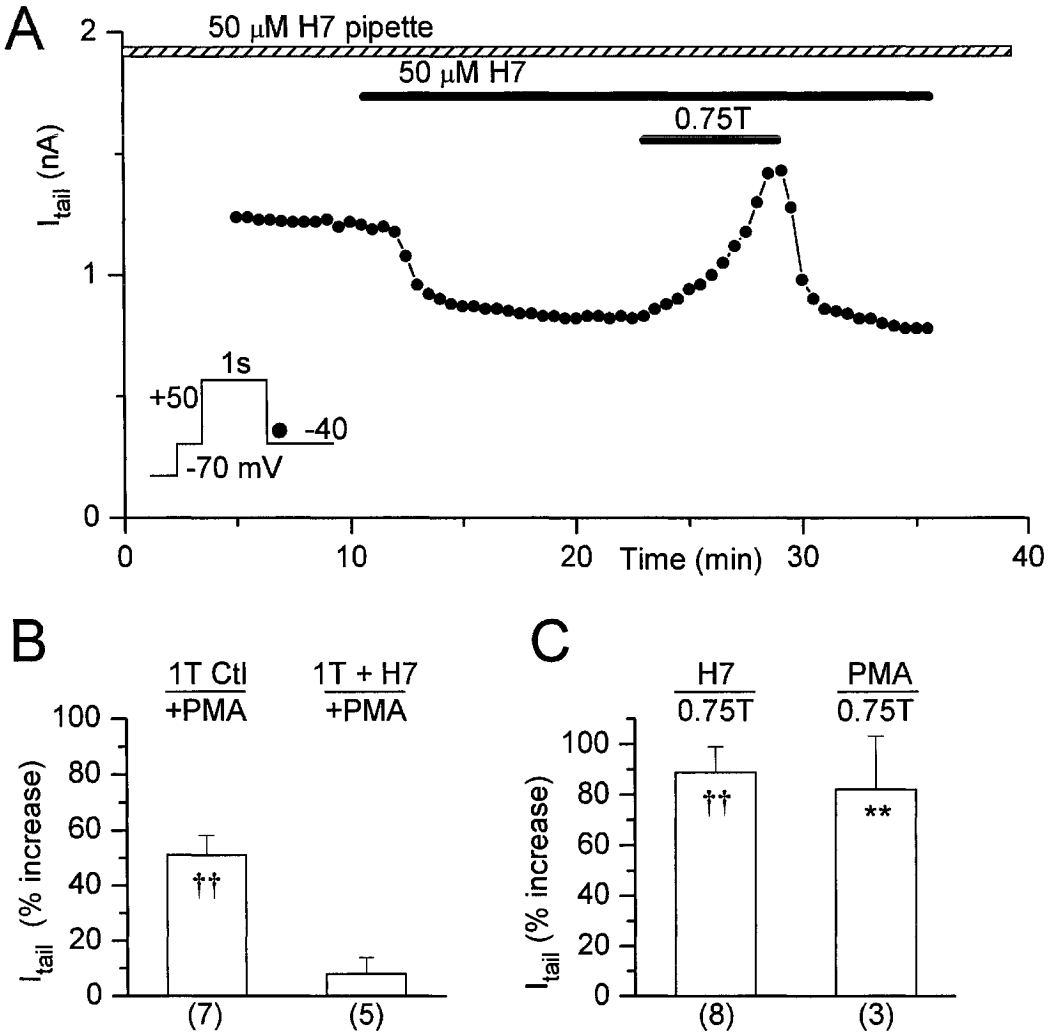

Figure 14: Lack of effect of PKC-inhibitor H7 and PKC-activator PMA on the stimulation of I_{Ks} by hyposmotic solution. All myocytes were dialyzed with 50 μM H7 pipette solution and pretreated with 50 μM H7. (A) Time course of changes in the amplitude of the I_{Ks} tail recorded from a representative myocyte. (B) Summary of data indicating that 10–15 pretreatment with external H7 (1T + H7) antagonized stimulation of I_{Ks} by 0.1 μM PMA. PMA was applied for 10 min, and percentage increases are referenced to pre-PMA current amplitudes. (C) Stimulation of I_{Ks} by hyposmotic solution in H7-pretreated and PMA-pretreated myocytes. Percentage increases are referenced to pre-0.75T current amplitudes. †† $p < 0.001$, ** $p < 0.05$, paired t-tests. Numbers of myocytes in parentheses.

Experiments with PMA

In agreement with earlier studies (e.g., Tohse *et al.*, 1990; Asai *et al.*, 1996), 0.1 μM PMA increased the amplitude of basal I_{Ks} by a significant 51 $\pm$ 7% (n = 7). It seems likely that this stimulation was mediated by PKC because the PMA effect was reduced to an insignificant 8 $\pm$ 6% (n = 5) in H7-pretreated myocytes (Figure 14B). In trials on three PMA-treated myocytes, hyposmotic PMA solution increased the amplitude of I_{Ks} by 82 $\pm$ 16% ($p < 0.05$) (Figure 14C).

Experiments with Bis

Bis is a highly selective and potent inhibitor of PKC (EC_{50} $\approx$70 nM) (Toullec *et al.*, 1991; Davies *et al.*, 2000). In earlier studies on PKC regulation of ion channels in cardiac cells, the inhibitor has been used at concentrations ranging from 0.03 μM (Duan *et al.*, 1995) to 0.1–0.2 μM (Ward and Giles, 1997; Lei *et al.*, 2000; Zhang *et al.*, 2003) and 1 μM (Kiehn *et al.*, 1998). In the present study, myocytes bathed with 1T solution were pretreated with 1 μM Bis for $\approx$10 min, and then exposed to hyposmotic Bis solution. I_{Ks} declined by about 20% during the first 5 min of pretreatment, and then stabilized. The records and data in Figure 15 indicate that the PKC inhibitor had little effect on the stimulation of I_{Ks} by 0.75T solution. In trials on six myocytes, the amplitude of the I_{Ks} tail increased by 68 $\pm$ 9% ($p < 0.001$).

3.4 Effects of PTK inhibitors

The purpose of the experiments reported in this section was to investigate the possible involvement of PTK in the acute regulation of I_{Ks} by determining the effects of

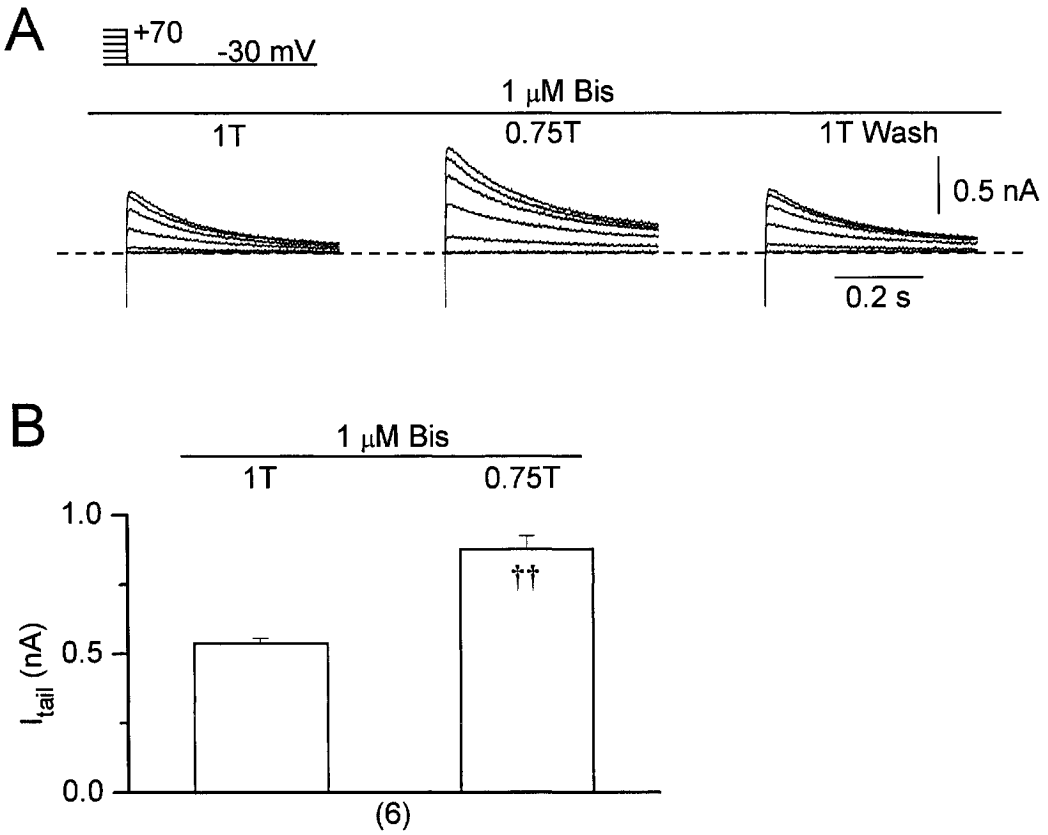

Figure 15: Hyposmotic stimulation of I_{Ks} in myocytes pretreated with 1 μM Bis. Six myocytes were pretreated with Bis for $\approx$10 min, exposed to hyposmotic Bis solution for 8 min, and then washed with isosmotic Bis solution. (A) Tail currents at -30 mV after 2-s depolarizations to more positive potentials. The currents were recorded after pretreatment with Bis (left), 7 min superfusion with 0.75T solution (middle), and 4 min recovery with 1T solution (right). (B) Summary of the results. The amplitude of I_{Ks} tails after 500-ms depolarization from -30 to $+50$ mV was measured just before (1T) and $\approx$7 min after switching to 0.75T solution. †† $p < 0.001$.

modulators of tyrosine phosphorylation on basal I_{Ks} and the responses of I_{Ks} to anisosmotic external solutions. The first subsection below describes the effects of four cell-permeable tyrphostin compounds; two of these, tyrphostins A25 and A23 (A25, A23), are broadspectrum inhibitors of PTK, whereas the other two, tyrphostins A1 and A63 (A1, A63) are relatively ineffective ("inactive") inhibitors of the kinase (Levitzki and Gazit, 1995; Davis *et al.*, 2001). The second subsection describes results obtained with the broadspectrum PTK inhibitor genistein and two inactive analogues, genistin and daidzein. The third subsection compares the effects of PP2, an inhibitor of the Src family of PTK, with those of inactive analogue PP3, and the final subsection describes the effects of AG1478, a relatively selective inhibitor of epidermal growth factor receptor (EGFR) PTK.

3.4.1 Broadspectrum PTK inhibitors A25 and A23, and negative controls A1 and A63

PTK inhibitor A25

Figure 16A–C shows records of the effects of 5, 30, and 150 μM A25 on time-dependent and tail I_{Ks} in myocytes bathed in 1T solution. Application of the tyrphostin for 8–10 min decreased the amplitude of the currents in a concentration-dependent manner, and the average data are well-described by the Hill equation (2.1) with an EC_{50} of 12.3 $\pm$ 2.1 μM and a coefficient of 0.99 $\pm$ 0.2 (Figure 16D). Application of 20 μM A25 did not significantly affect kinetics of activation and deactivation of I_{Ks} under 1T conditions (currents were elicited by 2-s pulses to +50 mV and fitted with double exponential functions; n =7).

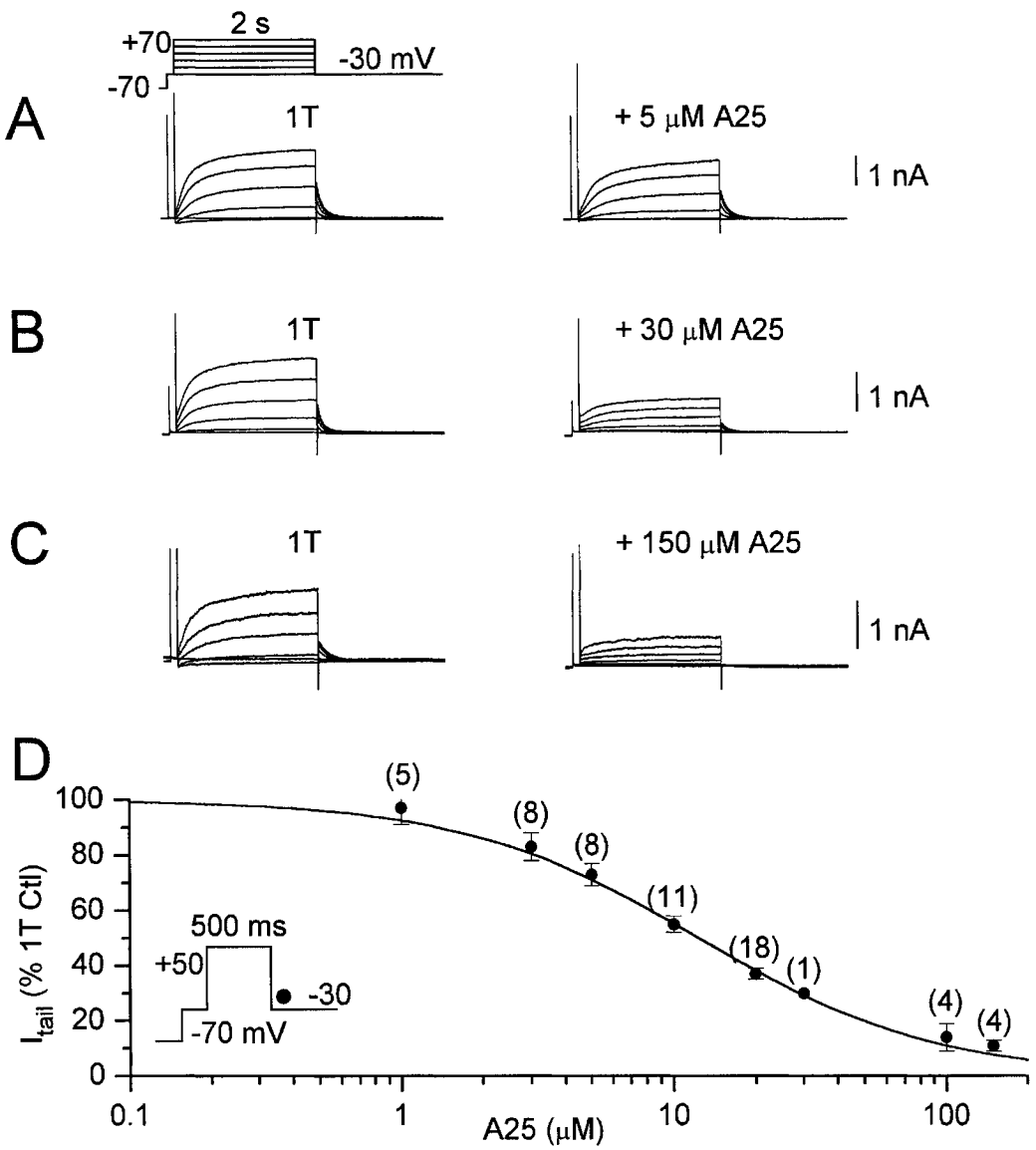

Figure 16: Concentration-dependent inhibition of basal I_{Ks} by A25. Myocytes bathed in 1T solution were depolarized from −30 to +50 mV for 500 ms at 0.1 Hz except for determination of 2-s I–V relationships just before (1T) and 8–10 min after exposure to a single concentration of A25. (A–C) Records obtained on I–V determinations in representative myocytes. (D) Concentration–response relationship. The amplitude of I_{Ks} was measured from tail currents elicited after 500-ms depolarizations from −30 to +50 mV. The Hill equation (2.1) fitting the data has an EC_{50} of 12.3 ± 2.1 μM and a coefficient of 0.99 ± 0.2. Numbers of myocytes in parentheses.

Similar experiments were performed on myocytes with I_{Ks} stimulated by superfusion with hyposmotic solution. The time courses of changes in the amplitude of tail I_{Ks} measured in two representative experiments are shown in Figure 17. In the first of these, the myocyte was bathed with 0.75T solution, and 20 μM A25 reduced the stimulated current by $\approx$70%, in a reversible manner (Figure 17A). In the second experiment, the myocyte was bathed in 0.5T solution, and 30 μM A25 reduced the stimulated current by $\approx$80% (Figure 17B). Overall, the inhibition of hyposmotic-stimulated I_{Ks} by A25 was well described by the Hill equation (2.1) with an EC_{50} of 15.8 $\pm$ 1.6 μM and a coefficient of 0.98 $\pm$ 0.1 (Figure 17C).

The foregoing results suggested that pretreatment of myocytes with A25 would affect the degree of stimulation of I_{Ks} by hyposmotic solution. The families of records in Figure 18A and B illustrate that pretreatment with low (e.g., 3 μM) concentrations of the drug had little effect on the degree of stimulation induced by hyposmotic solution, whereas pretreatment with higher (e.g., 20 μM) concentrations strongly antagonized the stimulation. The results obtained from 41 pretreated myocytes are summarized in the concentration–response plot of Figure 18C. The data are well described by the Hill equation (2.1) with an EC_{50} of 8.9 $\pm$ 1.2 μM and a coefficient of 1.09 $\pm$ 0.1.

The A25 concentration-response data raised the question of whether the myocytes that were pretreated with $\geq$10 μM A25 were, coincidentally, poor responders to hyposmotic solution. To address that concern, myocytes pretreated with 20 μM A25 were exposed to hyposmotic A25 solution as above, washed for 6 min with drug-free isosmotic solution, and then exposed to drug-free hyposmotic solution. The data obtained from one of these experiments are shown in Figure 19A. In this myocyte,

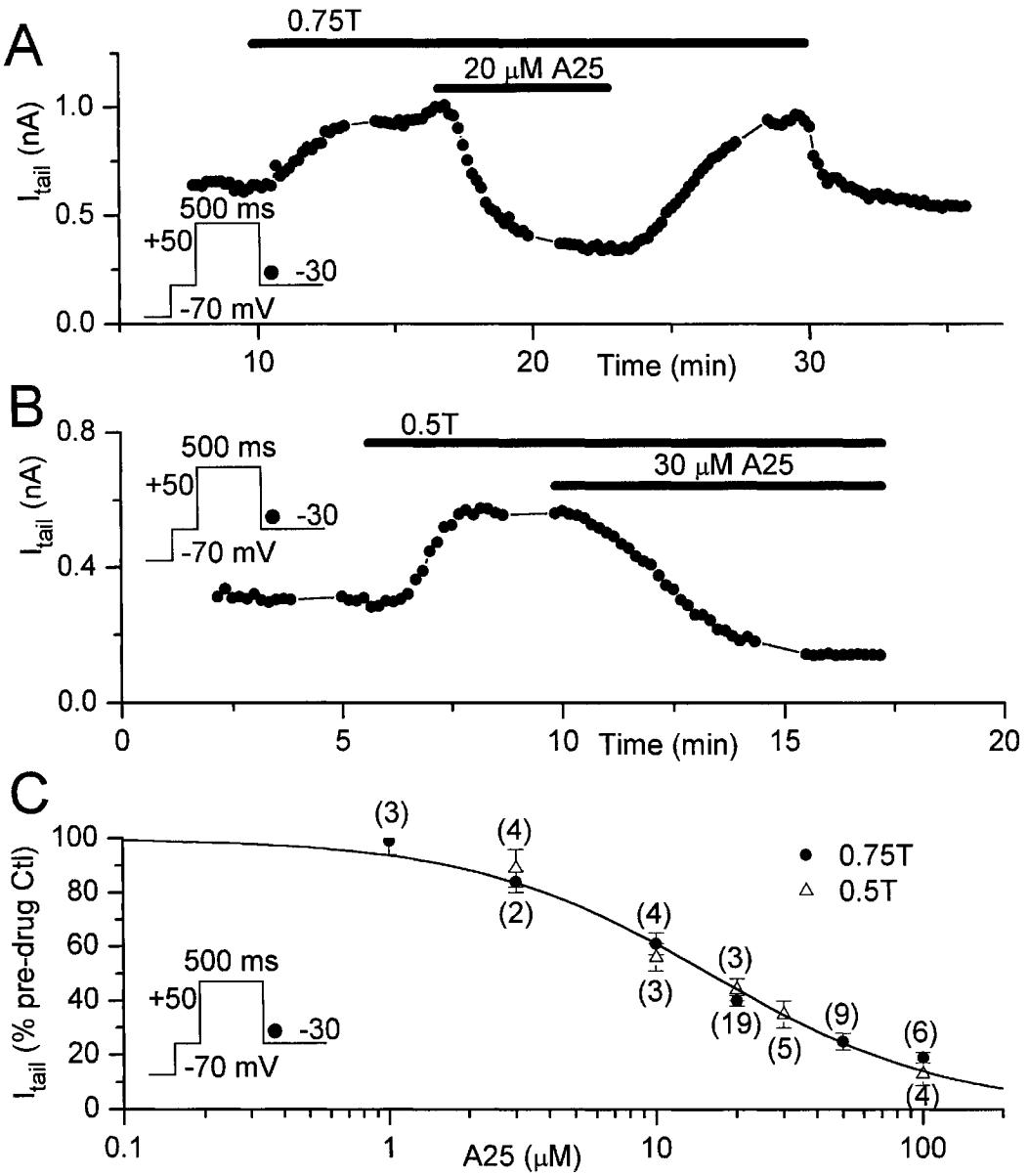

Figure 17: Inhibition of hyposmotic-stimulated I_{Ks} by A25. (A) Inhibition of 0.75T-stimulated I_{Ks} tail by 20 μM A25. (B) Inhibition of 0.5T-stimulated I_{Ks} by 30 μM A25. (C) Dependence of inhibition of hyposmotic-stimulated I_{Ks} on the concentration of A25. The Hill equation (2.1) fitting the data has an EC_{50} of 15.8 $\pm$ 1.6 μM and a coefficient of 0.98 $\pm$ 0.1. Numbers of myocytes in parentheses.

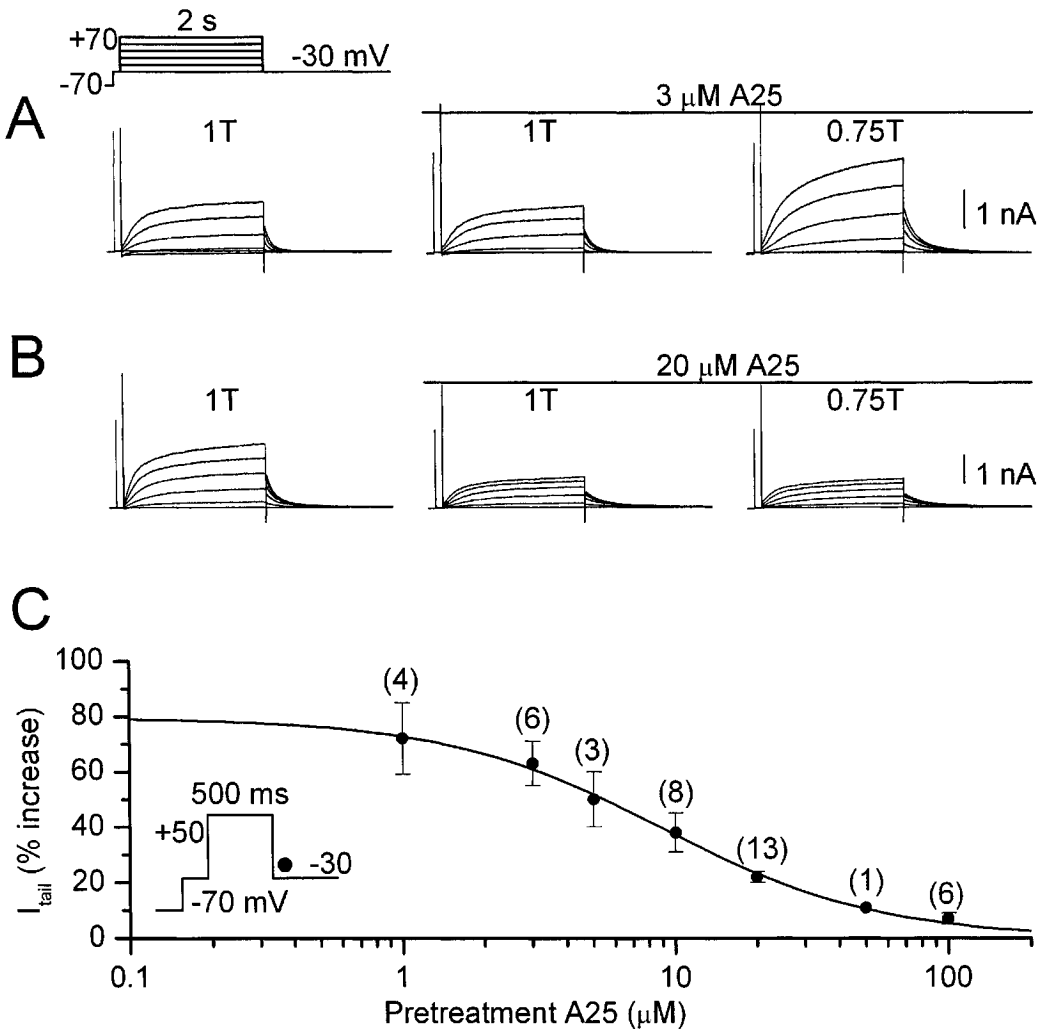

Figure 18: Antagonistic effect of A25 on the stimulation of I_{Ks} by hyposmotic solution. Myocytes bathed with 1T solution were treated with a single concentration of A25 for 6–8 min, and then exposed to hyposmotic A25 solution for 5–6 min. (A, B) Representative results obtained with 3 and 20 μM A25. (C) Concentration–response relationship. The percentage increase in I_{Ks} tail amplitude induced by hyposmotic solution was calculated by reference to the amplitude at the end of the isosmotic pretreatment. The data are fitted with a Hill equation (2.1) that has an EC_{50} of 8.9 $\pm$ 1.2 μM and coefficient of 1.09 $\pm$ 0.1. Numbers of myocytes in parentheses.

the pretreatment with A25 reduced the amplitude of I_{Ks} by 57%, hyposmotic A25 solution increased the (inhibited) current by 20%, washout with 1T solution restored the current to 85% of its pre-A25 amplitude, and subsequent exposure to hyposmotic solution increased the (restored) current by 66%. In seven experiments of this type, the percentage increases in I_{Ks} tail amplitude elicited by hyposmotic solution were 24 ± 6% in the presence of A25, and 70 ± 7% after washout of the drug ($p < 0.001$ versus A25) (Figure 19A).

To my knowledge, the only non-hyposmotic stimuli that can elicit a large increase in the amplitude of I_{Ks} are those that stimulate the cAMP–PKA pathway. To determine whether A25 antagonizes stimulation via the latter pathway, myocytes bathed in 1T solution were pretreated with 20 μM A25 and then co-treated with a relatively low (0.2 μM) concentration of forskolin (FSK). The time course of changes in the amplitude of I_{Ks} (Figure 19B) illustrates that A25 did not prevent stimulation by low FSK. In fact, the increase in I_{Ks} produced by FSK in A25-pretreated myocytes (54 ± 8%; n = 7; $p < 0.001$) was not significantly different than that observed in control myocytes (52 ± 6%; n = 7) (Figure 19B).

PTK inhibitor A23

A23 was a more potent inhibitor of I_{Ks} than was A25. As indicated by the example data in Figure 20A, a 1-μM concentration of A23 reduced the amplitude of the basal current by ≈20%. Higher concentrations had more pronounced effects, as indicated by the data shown in Figure 20B. In this experiment, application of 20 μM A23 reduced the amplitude of the current by more than 80%; the inhibition was fully reversible, but only after prolonged washout of the drug. Similar experiments were performed on

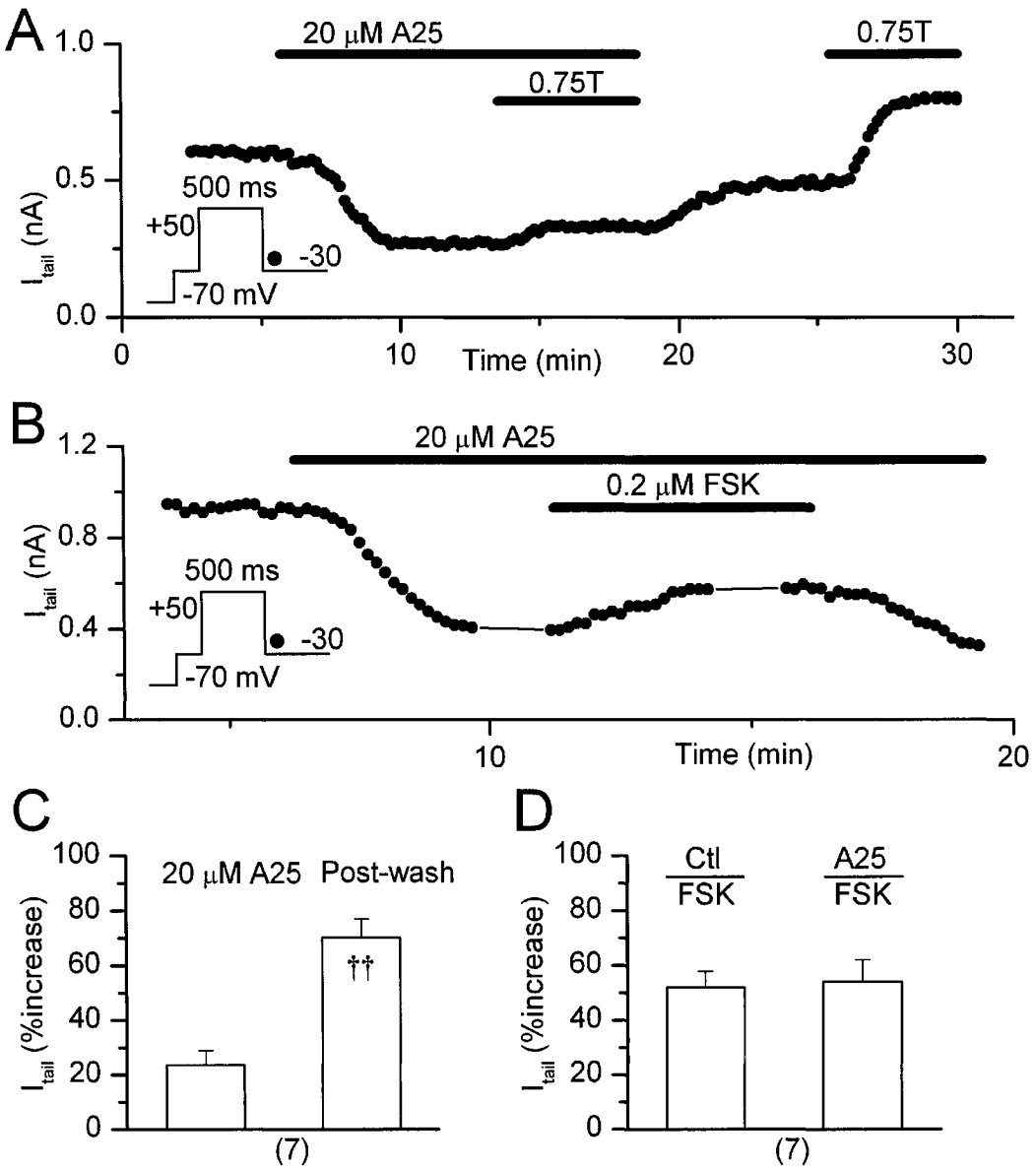

Figure 19: Responsiveness of myocytes treated with 20 μM A25. (A, C) Recovery of hyposmotic stimulation of I_{Ks} after washout of 20 μM A25. In C, the percentage increases in current amplitudes were calculated by reference to amplitudes measured just before exposures to hyposmotic solution. †† $p < 0.001$, paired t-test. (B, D) Response of control and A25 treated myocytes to 0.2 μM FSK. A25 had no effect on the stimulation of I_{Ks} by FSK.

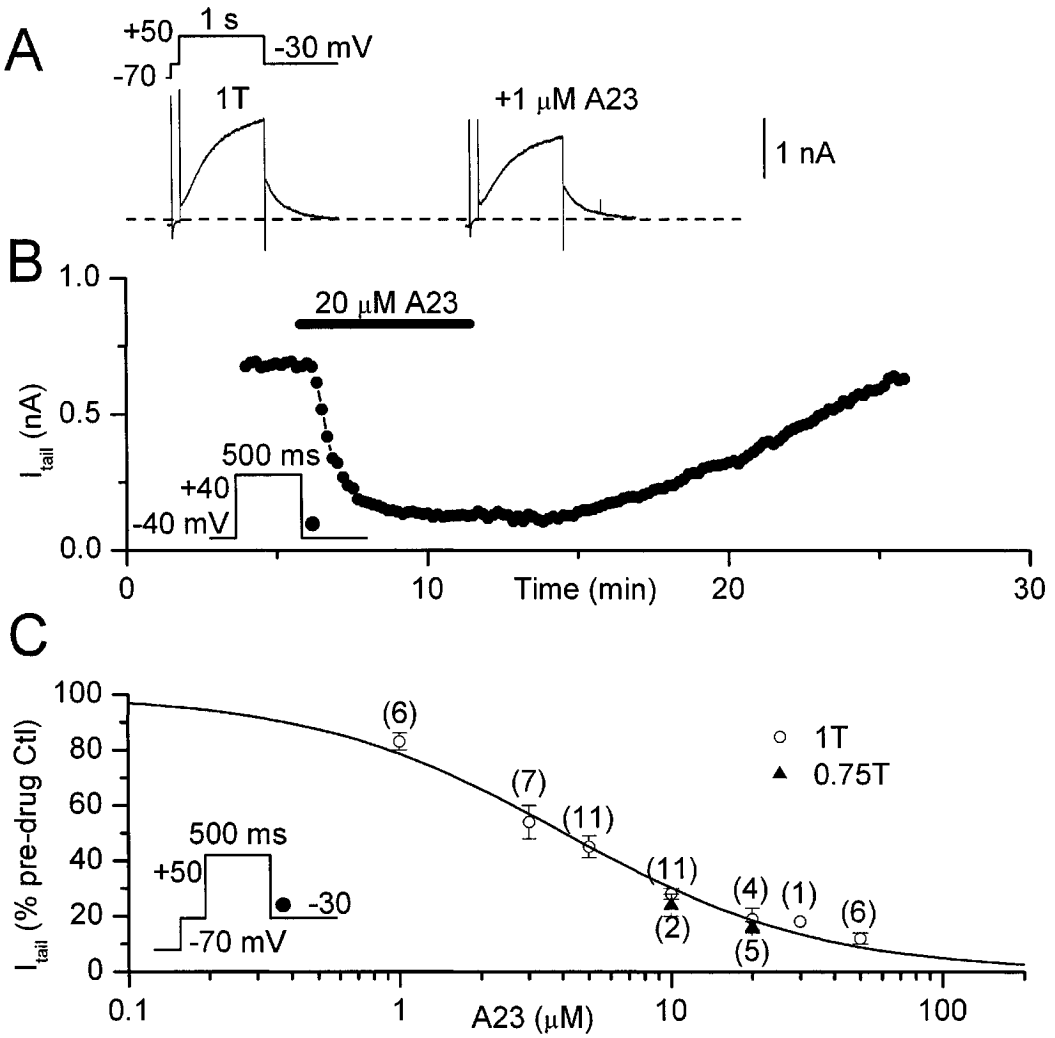

Figure 20: Effects of tyrphostin A23 pretreatment on I_{Ks} under 1T and 0.75T conditions. (A) Inhibition after an 8-min exposure to 1 μM A23. (B) Inhibition by 20 μM A23. (C) Concentration–response relationship. Each myocyte was treated with a single concentration of A23 under either 1T or 0.75T conditions. The Hill equation (2.1) fitting the data has an EC_{50} of 4.1 $\pm$ 0.6 μM and a coefficient of 0.93 $\pm$ 0.1. Numbers of myocytes in parentheses.

thirty-six myocytes superfused with 1T solution, and on seven myocytes superfused with 0.75T solution. As indicated by the data and curve shown in Figure 20C, there was a close correspondence between the 1T and 0.75T data sets; the Hill equation (2.1) fitting them has an EC_{50} of 4.1 $\pm$ 0.6 μM and a coefficient of 0.93 $\pm$ 0.1 (Figure 20C).

To determine the effects of A23 on the stimulation of I_{Ks} by hyposmotic solution, myocytes were pretreated with A23 and then exposed to hyposmotic A23 solution. As shown by the results in Figure 21, pretreatment with 3 and 10 μM A23 markedly depressed the stimulatory response to hyposmotic solution.

PTK-inactive tyrphostins A1 and A63

Tyrphostin A1 (A1) and tyrphostin A63 (A63) (Gazit *et al.*, 1989) are reported to have little inhibitory effect on the activity of PTK, and are therefore often employed as negative controls in experiments with A25 and A23 (Davis *et al.*, 2001). In the present study, high (50–100 μM) concentrations of A1 and A63 were used for comparison with the effects of A25 and A23.

Figure 22A illustrates the data obtained when myocytes bathed with 0.75T solution were treated with 100 μM A1. If anything, the inactive analogue had a small stimulatory effect on I_{Ks}. Overall, 100 μM A1 had no significant effect on I_{Ks} in myocytes bathed with 1T or 0.75T solution (n = 7 each) (Figure 22C). The results obtained with 50 μM A63 were similar to those obtained with A1 (Figure 22B, D).

The lack of effect of "PTK-inactive" tyrphostins on 0.75T-stimulated I_{Ks} suggests that the inhibitory effects of A23 and A25 were related to an inhibition of PTK. As described at a later point below, this postulate is supported by the results of

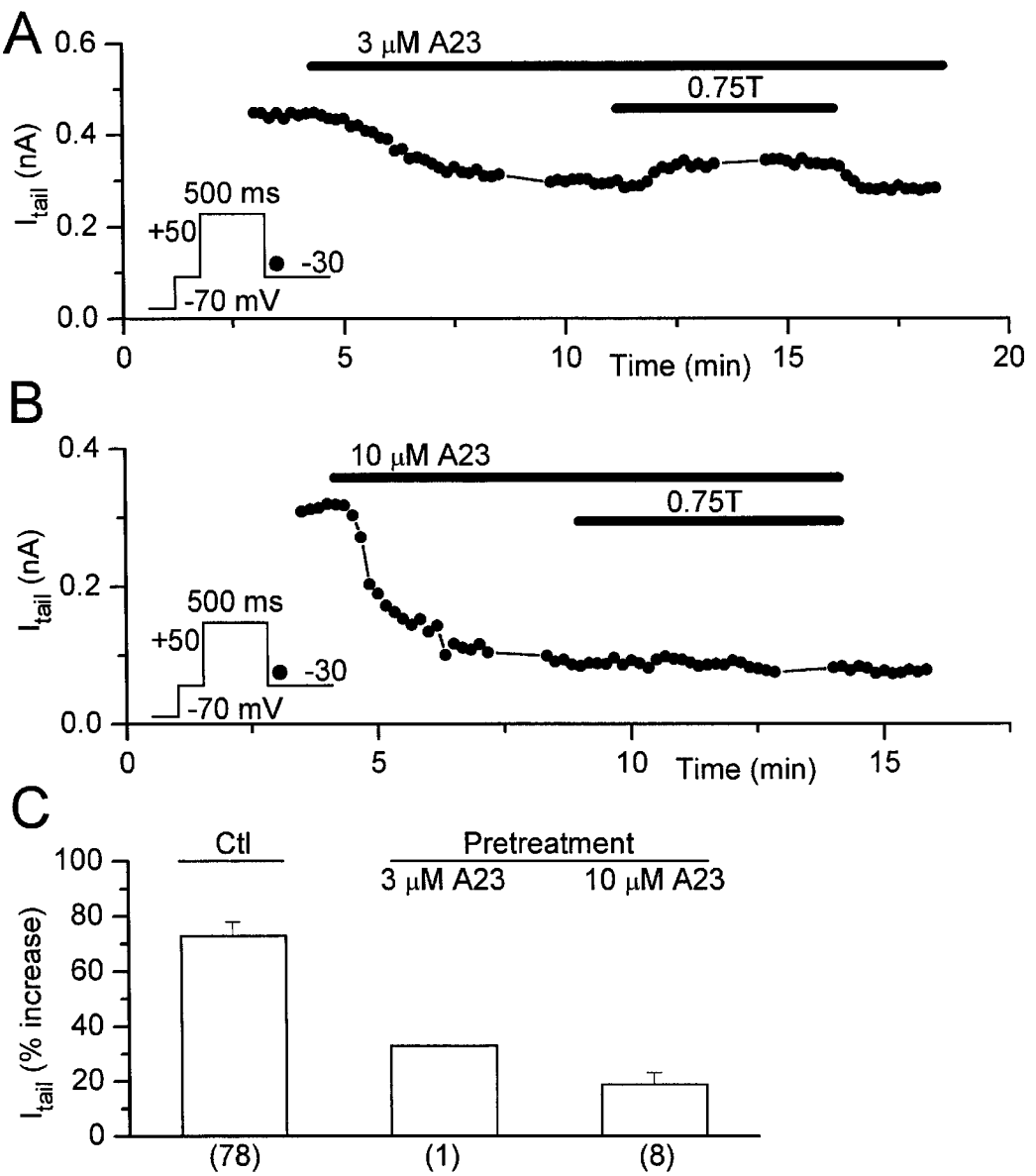

Figure 21: Inhibitory effect of A23 on the stimulation of I_{Ks} by hyposmotic solution. (A, B) Results obtained from representative myocytes pretreated with A23 prior to exposure to 0.75T solution. (C) Comparison of the results obtained in control myocytes (see Figure 7) with those obtained in A23-pretreated myocytes. Numbers of myocytes in parentheses.

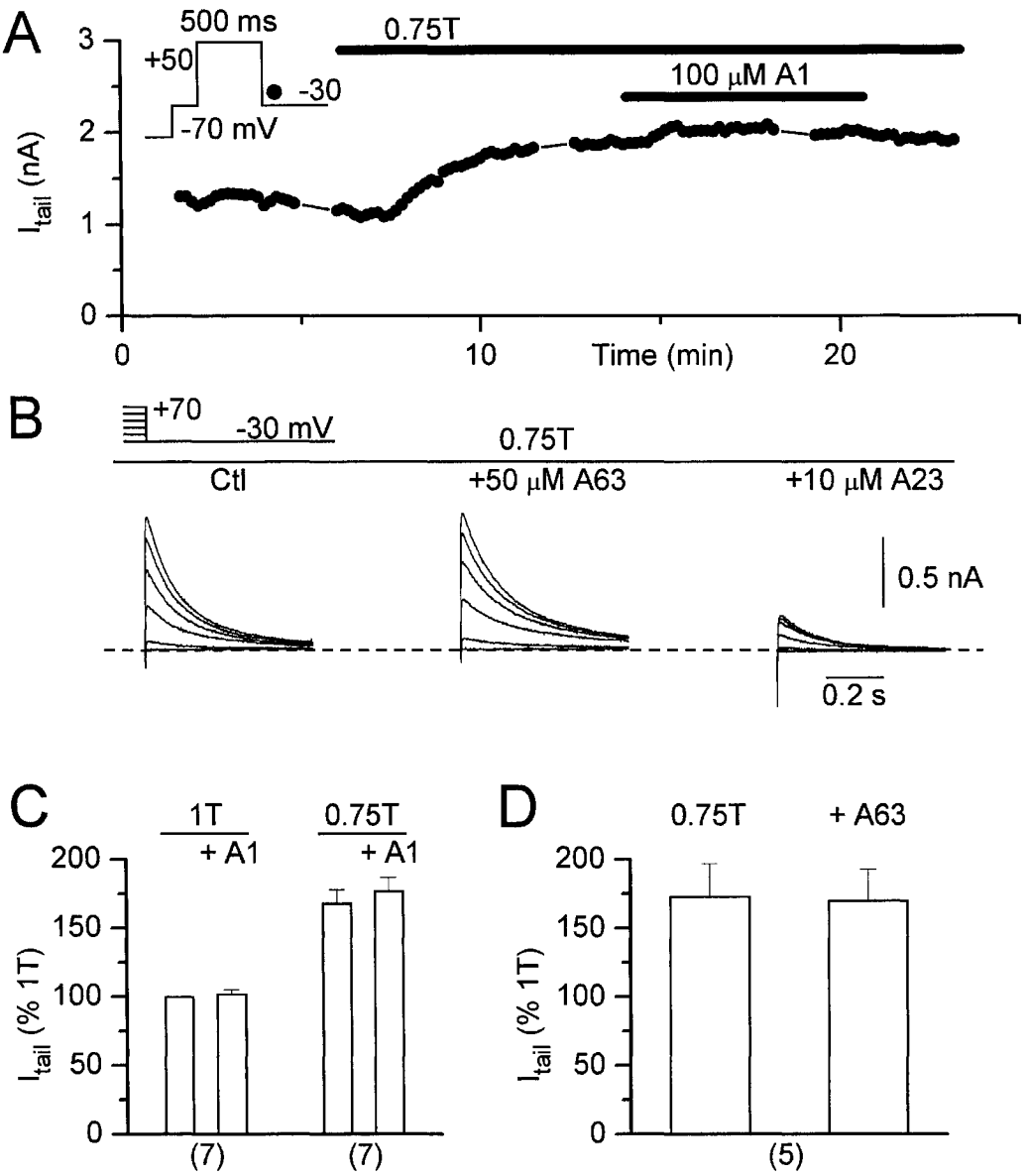

Figure 22: Lack of effect of tyrphostins A1 and A63 on I_{Ks}. (A) Time course of the amplitude of tail I_{Ks} from a representative myocyte superfused with 0.75T solution and then treated with 100 μM A1. (B) Records of I_{Ks} tails showing relative inactivity of 50 μM A63 versus 10 μM A23. The sets of traces were obtained at $\approx$6 min intervals. (C) Summary of the effects of 100 μM A1 on the amplitude of I_{Ks} in myocytes superfused with either 1T (left) or 0.75T (right) solution. (D) Summary of the effects of 50 μM A63 on I_{Ks} in myocytes bathed with 0.75T solution. Measurements of I_{Ks} in C and D were taken just before and 6–8 min after addition of tyrphostin. Numbers of myocytes in parentheses.

experiments with inhibitors of phosphotyrosyl phosphatase (PTP).

3.4.2 Broadspectrum PTK inhibitor genistein, and inactive analogues daidzein and genistin

The isoflavonoid genistein is a broad spectrum PTK inhibitor (Akiyama *et al.*, 1987) that has been shown to suppress ionic currents carried by L-type Ca^{2+} channels (Liu and Sperelakis, 1997; Strauss *et al.*, 1997; Ogura *et al.*, 1999), volume-sensitive Cl^- channels (Sorota, 1995; Crepel *et al.*, 1998; Voets *et al.*, 1998; Shi *et al.*, 2002), and voltage-gated K^+ channels (Peretz *et al.*, 1999; Strauss *et al.*, 2002; Soliven *et al.*, 2003), most likely via mechanisms that involve an inhibition of PTK. In regard to possible action on cardiac I_{Ks}, Hool *et al.* (1998) and Matsubayashi *et al.* (1999) have reported that genistein inhibits basal I_{Ks} in guinea pig ventricular myocytes. In addition, Zhou *et al.* (1997) have reported that I_{Ks} in canine ventricular myocytes was stimulated by 0.6T hyposmotic solution, and that the stimulation was antagonized by genistein. On the other hand, Kubota *et al.* (2002) have recently concluded that the drug (50 μM) does not inhibit the hyposmotic stimulation of current carried by expressed KCNQ1/KCNE1 channel subunits.

Figure 23A, B shows the tail currents that were recorded from a representative myocyte that was exposed to 50 μM genistein for 8 min. The isoflavonoid decreased the amplitude of the I_{Ks} tail by $\approx$40%, and this inhibitory action was fully reversed after a washout period with drug-free solution. Similarly, the inhibitory actions of higher concentrations of genistein were fully reversible (e.g., Figure 23B).

The degree of inhibition induced by genistein was dependent on the concentration of the drug, and data gathered from experiments with concentrations ranging from 1

to 200 μM are well-described by the Hill equation (2.1) with an EC_{50} of 64 $\pm$ 3.8 μM and a coefficient of 0.8 $\pm$ 0.04 (Figure 23C).

Genistin and daidzein are structural analogues of genistein (Akiyama and Ogawara, 1991; Lavens *et al.*, 1992). For this reason, they are commonly used as negative controls in studies on the actions of genistein (Ogura *et al.*, 1999; Shibata *et al.*, 1999; Okamoto *et al.*, 2001). As shown by the concentration–response data in the upper right quadrant of Figure 23C, the amplitude of I_{Ks} was unaffected by genistin at concentrations up to 200 μM (see also Figure 23B). Although daidzein (20, 50, 150 μM) had a concentration-dependent inhibitory effect on the current, its potency was considerably weaker than that of genistein (Figure 23C). One of the previous studies (Zhou *et al.*, 1997) reported lack of effects of daidzein on swelling-activated I_{Ks}.

To evaluate the inhibitory effects of genistein on the stimulation of I_{Ks} by hyposmotic solution, myocytes bathed with 1T solution were pretreated with 50 μM genistein and then exposed to 0.75T solution in the continued presence of the drug. In eleven experiments, the hyposmotic exposure increased the amplitude of the current 36 $\pm$ 4%, a value that is substantially smaller than that observed in control myocytes ($p < 0.001$).

3.4.3 Src-selective PP2 and inactive analogue PP3

Tyrphostins A23 and A25 inhibit Src kinase *in vitro* with EC_{50} of 11 and 10 μM respectively (Agbotounou *et al.*, 1994). In addition, A25 inhibits human Src with EC_{50} of $\approx$20 μM and both GST and A25 inhibit angiotensin-induced Src-mediated contractions in porcine coronary tissue with EC_{50} of $\approx$10 μM (Oda *et al.*, 1999). To elucidate the role of Src family kinase in the modulation of I_{Ks}, a potent inhibitor

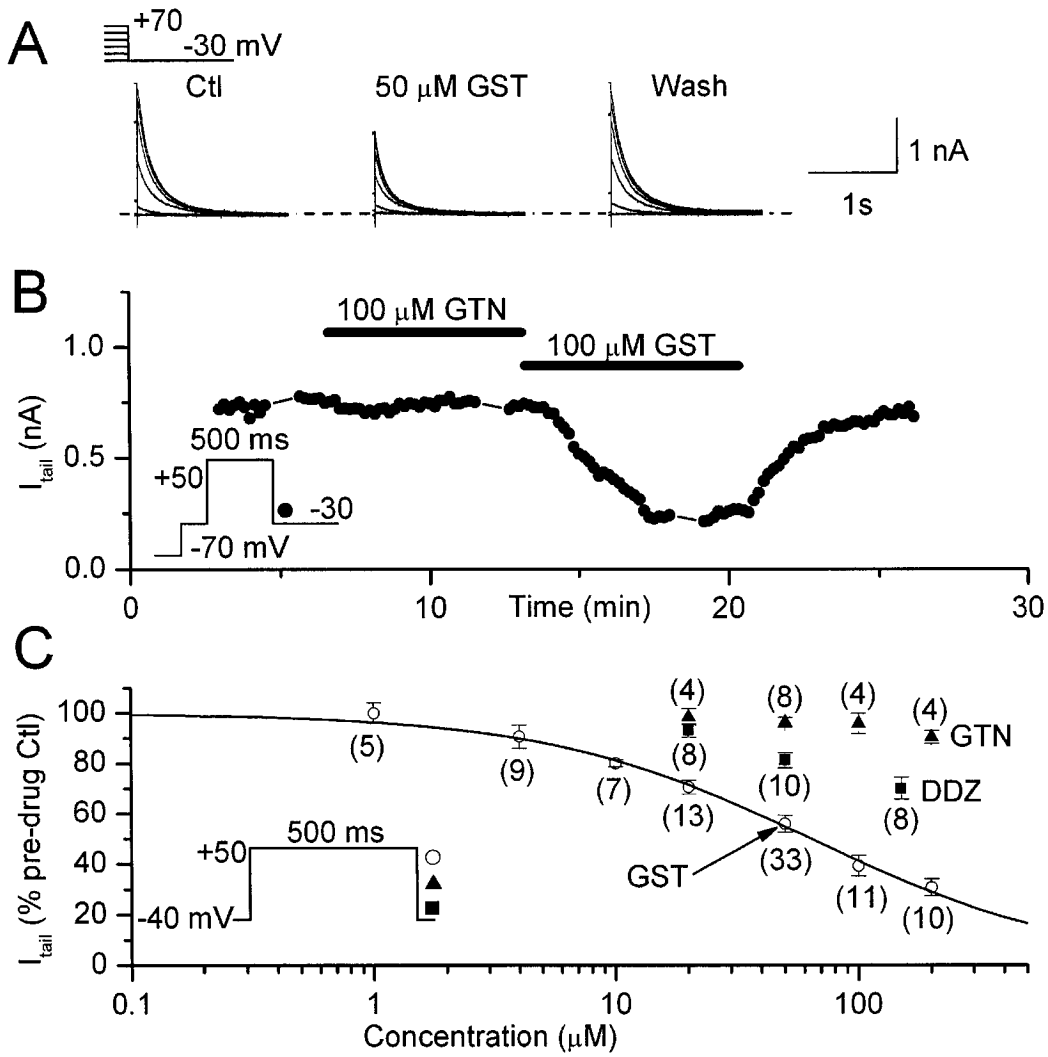

Figure 23: Effects of genistein, daidzein, and genistin on basal I_{Ks}. (A, B) Examples of the inhibitory effects of 50 and 100 μM genistein (GST) on the amplitude of the I_{Ks} tail. (C) Summary of the concentration-dependent inhibitory effects of genistein (open circles), daidzein (filled squares), and genistin (filled triangles). The Hill equation (2.1) fitting the genistein data has an EC_{50} of 64 $\pm$ 3.8 μM and a coefficient of 0.8 $\pm$ 0.04. Numbers of myocytes in parentheses.

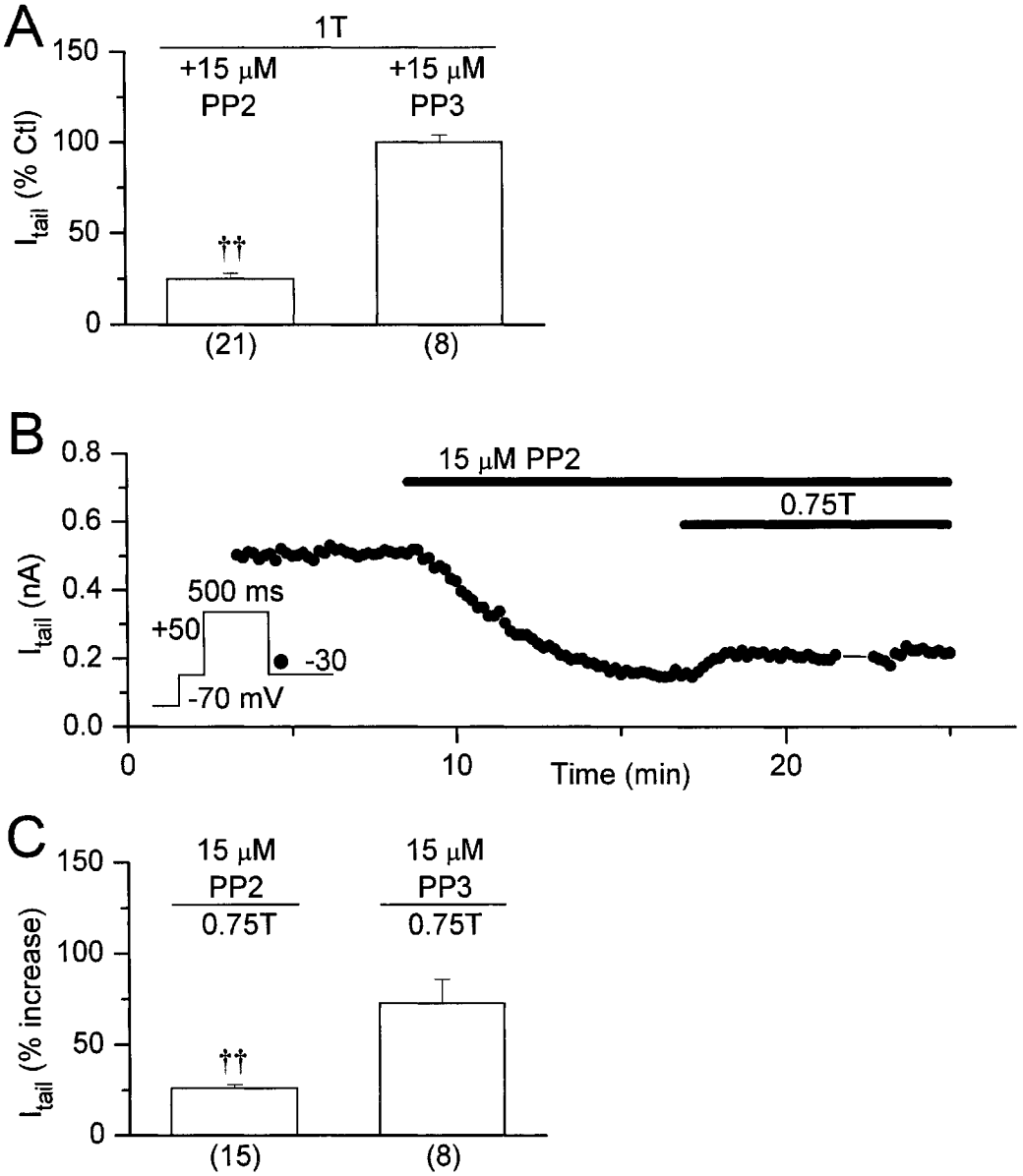

Figure 24: Effects of PP2 and PP3 on I_{Ks}. (A) Strong inhibition of basal I_{Ks} by 15 μM PP2, but not by 15 μM PP3. †† $p < 0.001$ versus control and PP3. (B) Weak stimulation of I_{Ks} by hyposmotic solution in a representative myocyte pretreated with PP2. (C) Summary of the data on hyposmotic stimulation of I_{Ks} in myocytes pretreated with PP2 and PP3. †† $p < 0.001$ versus PP3, unpaired t-test. Numbers of myocytes in parentheses.

of the Src family of PTK, PP2 (nanomolar EC_{50}: Hanke *et al.*, 1996) was employed. PP2 is commonly used to investigate the involvement of Src in the regulation of ion channel activity (e.g., MacFarlane and Sontheimer, 2000; Wang *et al.*, 2003; Du *et al.*, 2004). Myocytes were pretreated with 15 μM PP2 or 15 μM PP3 (negative control) and then exposed to 0.75T solution in the continued presence of the drug. The data presented in Figure 24 indicate that PP2 reduced the amplitude of basal I_{Ks} to 26 $\pm$ 3% (n = 21) of control value ($p < 0.001$), whereas PP3 had no effect on the current (100 $\pm$ 4% of control, n = 8).

In addition to its inhibitory action on basal I_{Ks}, PP2 markedly inhibited the stimulation of I_{Ks} by hyposmotic solution. In fifteen PP2-pretreated myocytes, exposure to hyposmotic solution only increased the amplitude of I_{Ks} by 25 $\pm$ 3%. By contrast, the increase in eight PP3-pretreated myocytes was a control-like 77 $\pm$ 13% ($p < 0.001$ versus PP2).

3.4.4 EGFR inhibitor AG1478

Recent studies indicate that EGFR is expressed and active in adult cardiac myocytes (e.g., Kudoh *et al.*, 1998; Haas *et al.*, 2000), and that EGFR may be involved in the modulation of cellular responses caused by acute changes in external osmolarity (Shen *et al.*, 2001; Rodriguez *et al.*, 2002; Abdullaev *et al.*, 2003; Du *et al.*, 2004). Tyrphostin A25 is a relatively potent inhibitor of EGFR, EC_{50} values of 1–3 μM (Gazit *et al.*, 1989; Partik *et al.*, 1999). Genistein inhibits EGFR in A431 cells with EC_{50} value of 111 μM (Akiyama and Ogawara, 1991). It is, therefore, possible that modulatory actions of genistein and tyrphostins A23 and A25 on I_{Ks} were due to the inhibition of EGFR.

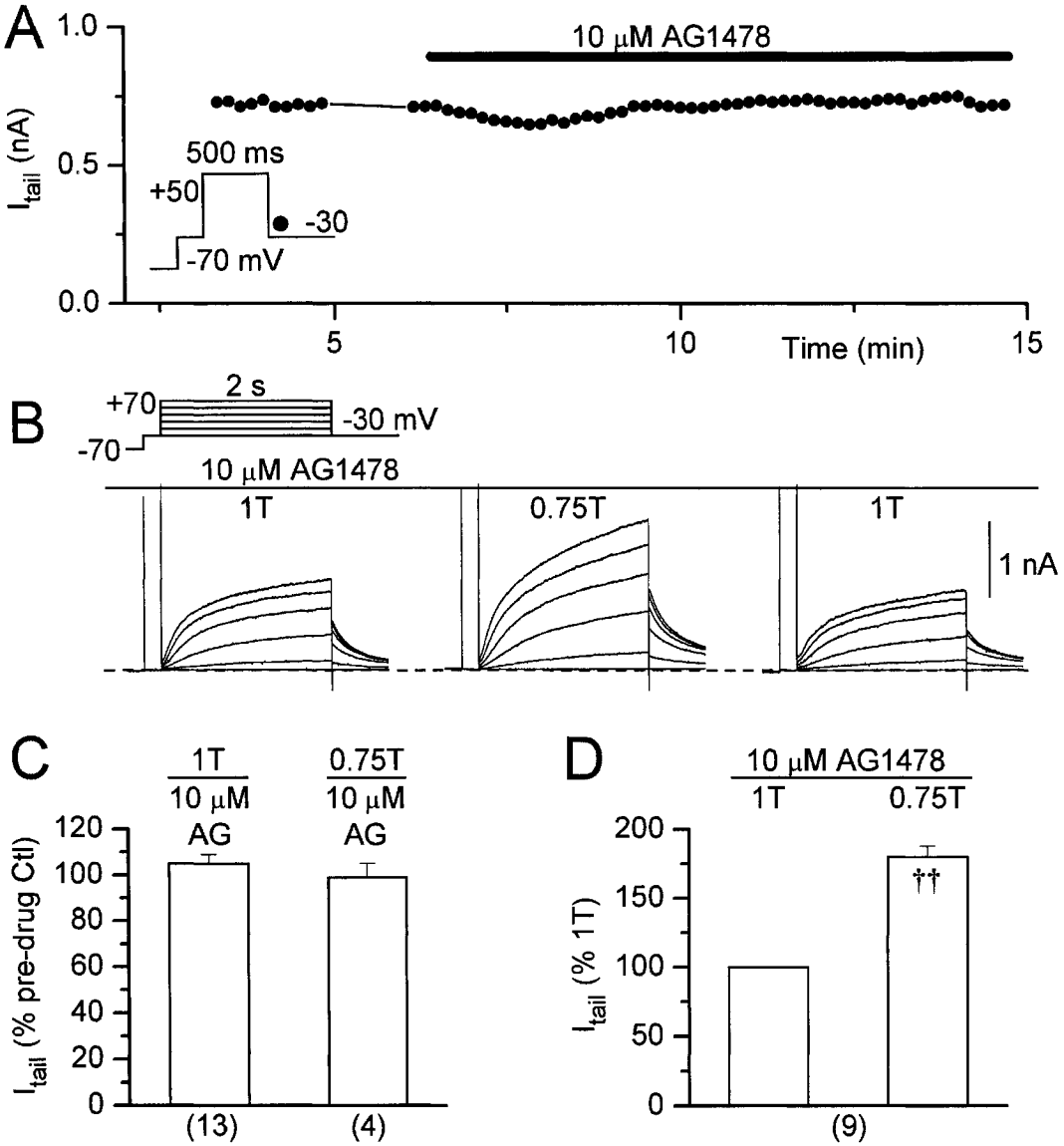

Figure 25: Lack of effect of 10 μM AG1478 on hyposmotic stimulation of I_{Ks}. (A) Time course of I_{Ks} tail amplitude recorded from a representative myocyte bathed in 1T solution and treated with AG1478. (B) Records obtained from a myocyte pretreated with AG1478 and then exposed to hyposmotic drug solution. Elapsed time between each set of records was $\approx$5 min. (C) Summary of results obtained in experiments like those in A from myocytes bathed in either 1T (left) or 0.75T (right) solution. (D) Summary of results obtained from experiments like those in B. †† $p <$ 0.001, paired t-test. Numbers of myocytes in parentheses.

To investigate whether EGFR was involved in the stimulation of I_{Ks} by hyposmotic solution, myocytes were pretreated with 10 μM tyrphostin AG1478 (AG1478) (EC_{50} $\simeq$3 nM for EGFR: Levitzki and Gazit, 1995; Liu *et al.*, 1999), and then exposed to hyposmotic drug solution. As indicated by the results shown in Figure 25, the exposure to hyposmotic solution resulted in an 80 $\pm$ 8% increase in the amplitude of the I_{Ks} tail (n = 8; $p < 0.001$). This finding suggests that EGFR is not in the signaling pathway leading to stimulation of I_{Ks} by hyposmotic swelling.

3.5 Effects of PTP inhibitors

The results described in section 3.4 raised the question of whether the decrease of I_{Ks} by PTK inhibitor might, to some degree, be reversed by an inhibition of tyrosine dephosphorylation. This possibility was investigated by studying the effects of two phosphotyrosyl phosphatase (PTP) inhibitors, sodium orthovanadate (Van) (Swarup *et al.*, 1982; Gordon, 1991) and bis(N,N-dimethylhydroxamido) hydroxooxovanadate (DMHV) (Cuncic *et al.*, 1999; Hsu *et al.*, 2003).

3.5.1 Control experiments

In the first two sets of control experiments, myocytes were bathed in either 1T or 0.75T solution and treated with a PTP inhibitor for 8–10 min. Van increased the amplitude of I_{Ks} tail by 6 $\pm$ 3% (n = 18; $p < 0.05$) under 1T conditions, and by 2 $\pm$ 5% (n = 4) under 0.75T conditions (Figure 26A), whereas DMHV had no significant effect under either 1T or 0.75T conditions (n = 4 each).

In the third set of control experiments, myocytes were pretreated with 1 mM

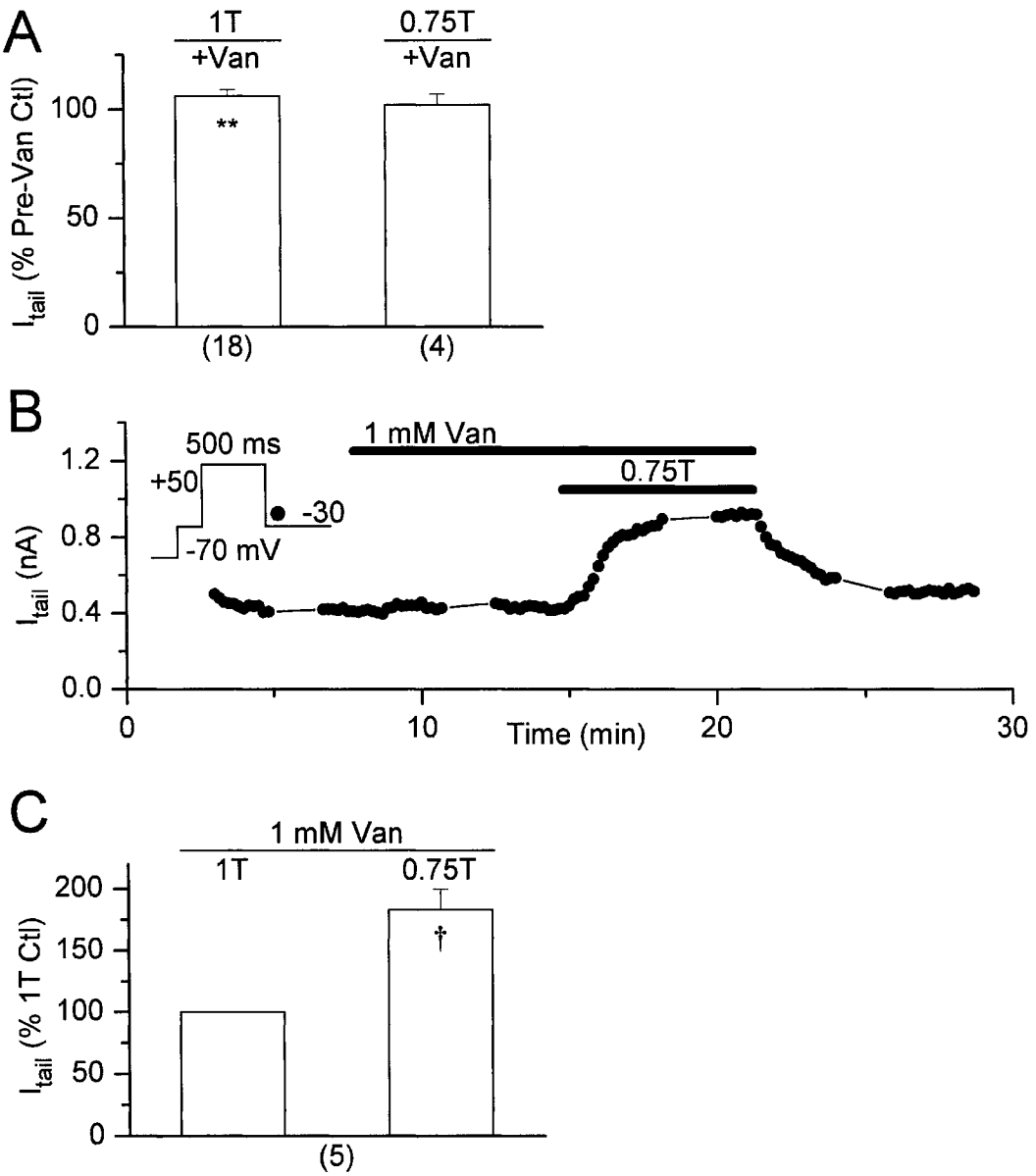

Figure 26: Effects of 1 mM Van on the amplitude of I_{Ks} under 1T and 0.75T conditions. (A) Little or no effect of Van (10 min) on I_{Ks} in myocytes bathed with 1T or 0.75T solution. ** $p < 0.05$, paired t-test. (B) Time course of I_{Ks} tail amplitude during pretreatment of a myocyte with 1 mM Van in 1T solution, and subsequent superfusion with hyposmotic Van solution. (C) Summary of the data obtained in experiments similar to that depicted in B. † $p < 0.01$, paired t-test. Numbers of myocytes in parentheses.

Van or 100 μM DMHV, and then exposed to 0.75T solution (e.g., Figure 26B). Van had no apparent effect on the stimulation of I_{Ks} by the hyposmotic solution; in five myocytes, the amplitude of the I_{Ks} tail increased by 83 $\pm$ 17% ($p < 0.01$) (Figure 26C). Similarly, DMHV had no apparent effect on hyposmotic stimulation (increase of 69 $\pm$ 7%; n = 4; $p < 0.001$).

3.5.2 Vanadate antagonism of PTK-inhibitor action on I_{Ks}

Possible interplay between PTK inhibition and PTP inhibition on the amplitude of I_{Ks} was investigated by determining the effects of Van or DMHV on I_{Ks} inhibited by pretreatment with PTK inhibitors.

Reversal of A25-induced inhibition

To test the effects of 1 mM Van on I_{Ks} inhibited by A25, myocytes were superfused with 0.75T solution and then exposed to 20 μM A25 for $\approx$5 min prior to the addition of Van. Figure 27A depicts the data obtained from a representative myocyte investigated with this protocol. The A25 treatment reduced the amplitude of the 0.75T-stimulated I_{Ks} by $\approx$55%, and the subsequent addition of 1 mM Van resulted in a complete reversal of this inhibition. Similar inhibition by 20 μM A25 in a different myocyte was almost completely reversed by 0.1 mM DMHV (Figure 27B).

In thirteen myocytes, the 0.75T-stimulated current was reduced to 42 $\pm$ 4% of control (0.75T) amplitude by 20 μM A25 ($p < 0.001$), and restored to 85 $\pm$ 5% of control amplitude by co-application of 1 mM Van ($p < 0.001$ versus A25) (Figure 27C). Similarly, DMHV increased the amplitude of I_{Ks} from 46 $\pm$ 6% to 91 $\pm$ 24% (n = 4; $p < 0.05$ versus A25) of control (0.75T) amplitude (Figure 27D).

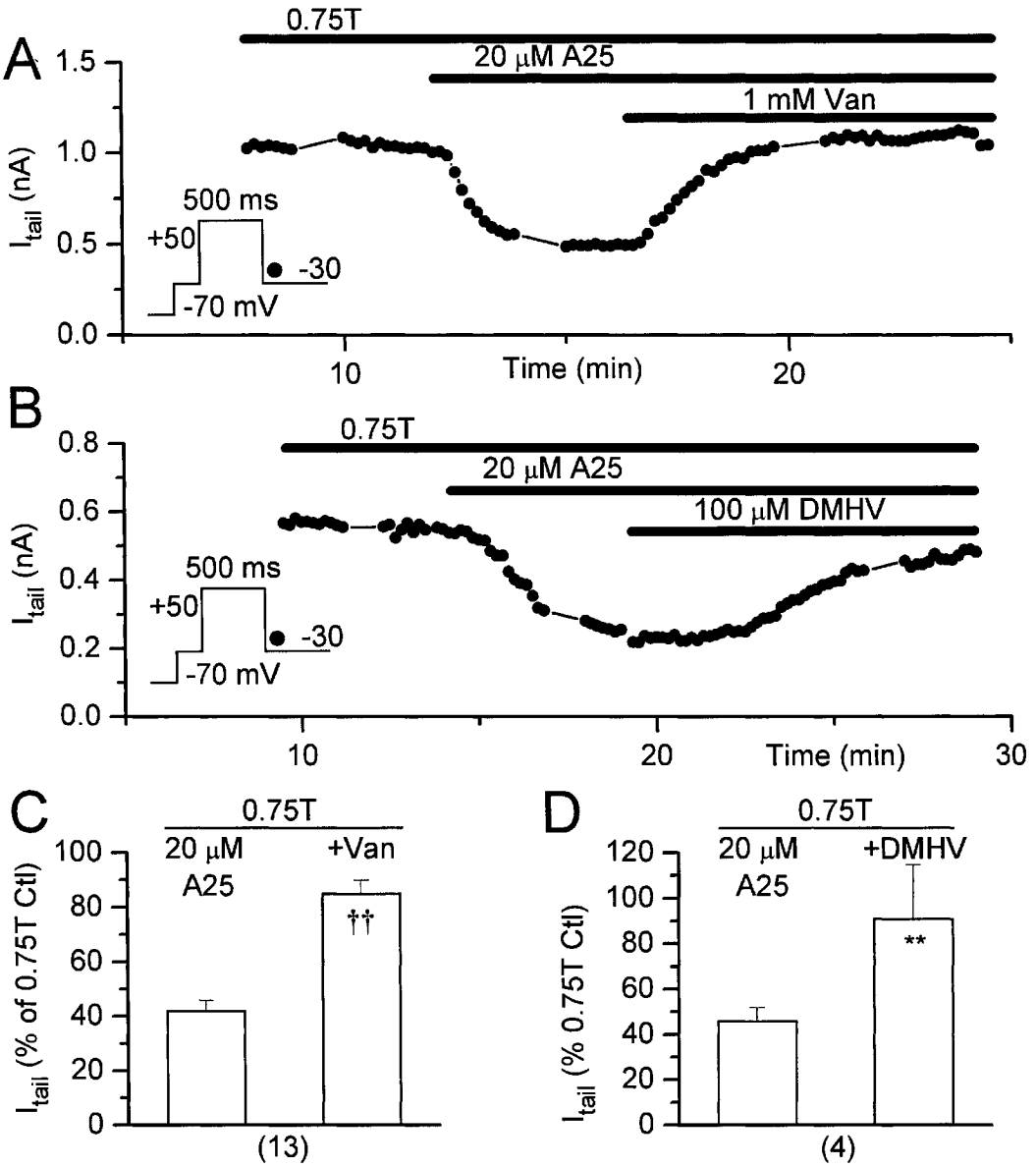

Figure 27: Reversal of A25-induced inhibition of I_{Ks} by 1 mM Van and 0.1 mM DMHV. (A) Time course of the amplitude of the I_{Ks} tail recorded from a representative myocyte bathed with 0.75T solution, and then treated with 20 μM A25 and 20 μM A25 + 1 mM Van. Note the marked reversible effect of Van. (B) Similar type of experiment with A25 and DMHV. (C) Summary of the data obtained from thirteen experiments as in A. †† $p < 0.001$ versus 0.75T + A25, paired t-test. (D) Summary of the data obtained from four experiments as in B. ** $p < 0.05$ versus 0.75T + A25, paired t-test. Numbers of myocytes in parentheses.

Reversal of A23- and genistein-induced inhibition

The reversal of A23-induced inhibition of I_{Ks} by PTP inhibitor was investigated under 1T conditions using 3–5 μM A23 and 1 mM Van. Exposure to A23 reduced the amplitude of the tail current to 50 $\pm$ 6% of control, and Van increased it to 98 $\pm$ 9% control (n = 5; $p < 0.001$ versus A23) (Figure 28A, C).

Van was also an effective antagonist of genistein action on I_{Ks} (e.g., Figure 28B). In nine experiments, 50 μM genistein reduced the amplitude of the I_{Ks} tail to 59 $\pm$ 2% control ($p < 0.001$), and co-application of Van restored it to 89 $\pm$ 3% control ($p < 0.001$ versus pre-Van value) (Figure 28C).

Reversal of very strong inhibition

The foregoing results suggest that when dephosphorylation is curtailed, residual PTK activity in the presence of 20 μM A25 promotes phosphorylation and strong restoration of I_{Ks}. If that is the case, stronger inhibition of PTK by application of a higher concentration of A25 ought to attenuate/prevent restoration of I_{Ks} by vanadate. As illustrated by the data in Figure 29A, 50 μM A25 markedly decreased the amplitude of the I_{Ks} tail, and subsequent co-application of 1 mM Van increased the tail amplitude, but not to the extent observed in the experiments with lower concentrations of A25. Similar results were obtained from two myocytes pretreated with 100 μM A25, and from three myocytes pretreated with 50 μM A23 (e.g., Figure 29B, C).

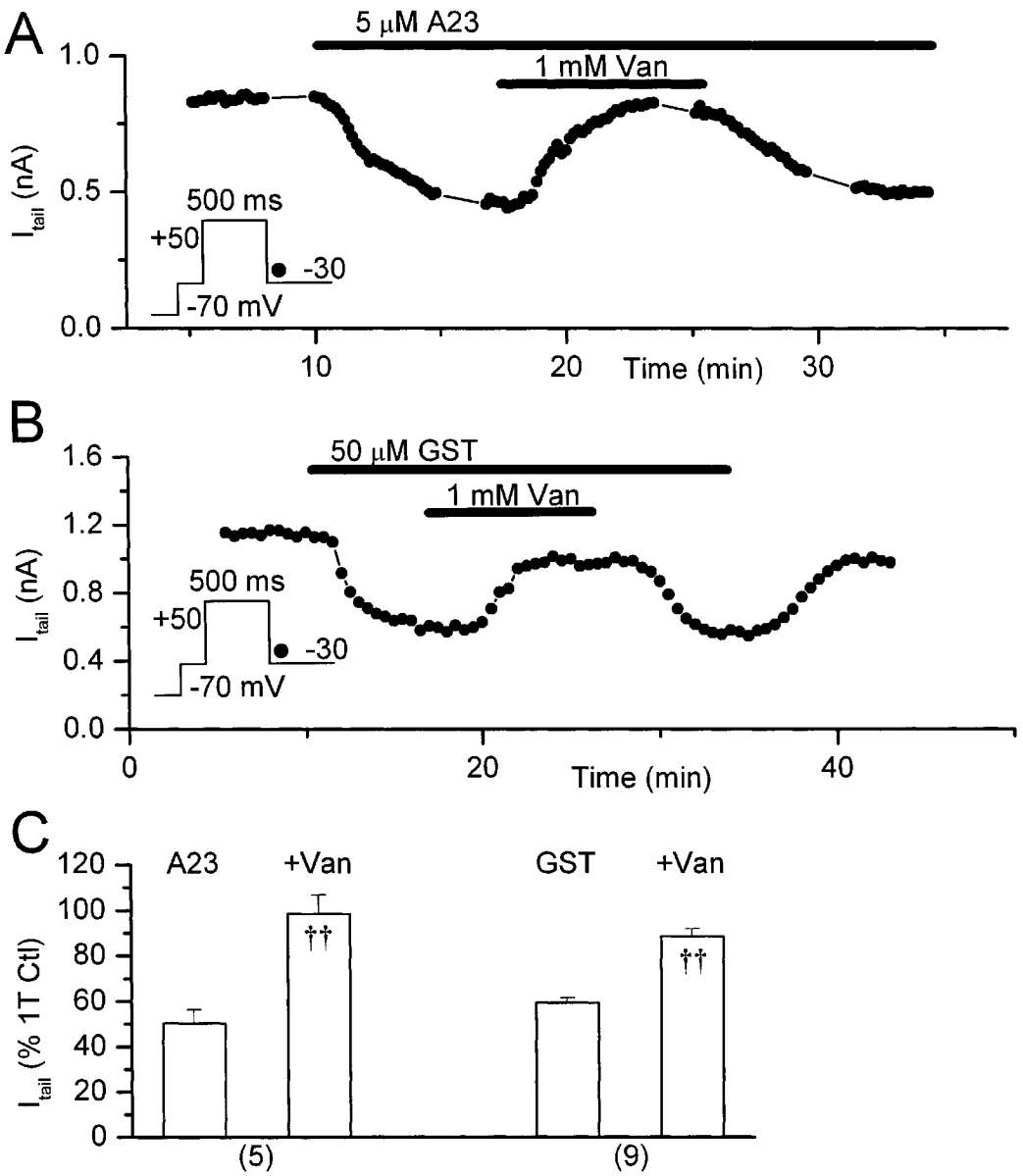

Figure 28: Reversal of A23- and genistein-induced inhibition of basal I_{Ks} by Van. (A) Time course of the amplitude of the I_{Ks} tail during application of 5 μM A23 and subsequent co-application of 1 mM Van to a representative myocyte. (B) Results of a similar experiment with 50 μM genistein. (C) Summary of the data. †† $p < 0.001$ versus pre-Van values, paired t-test. Numbers of myocytes in parentheses.

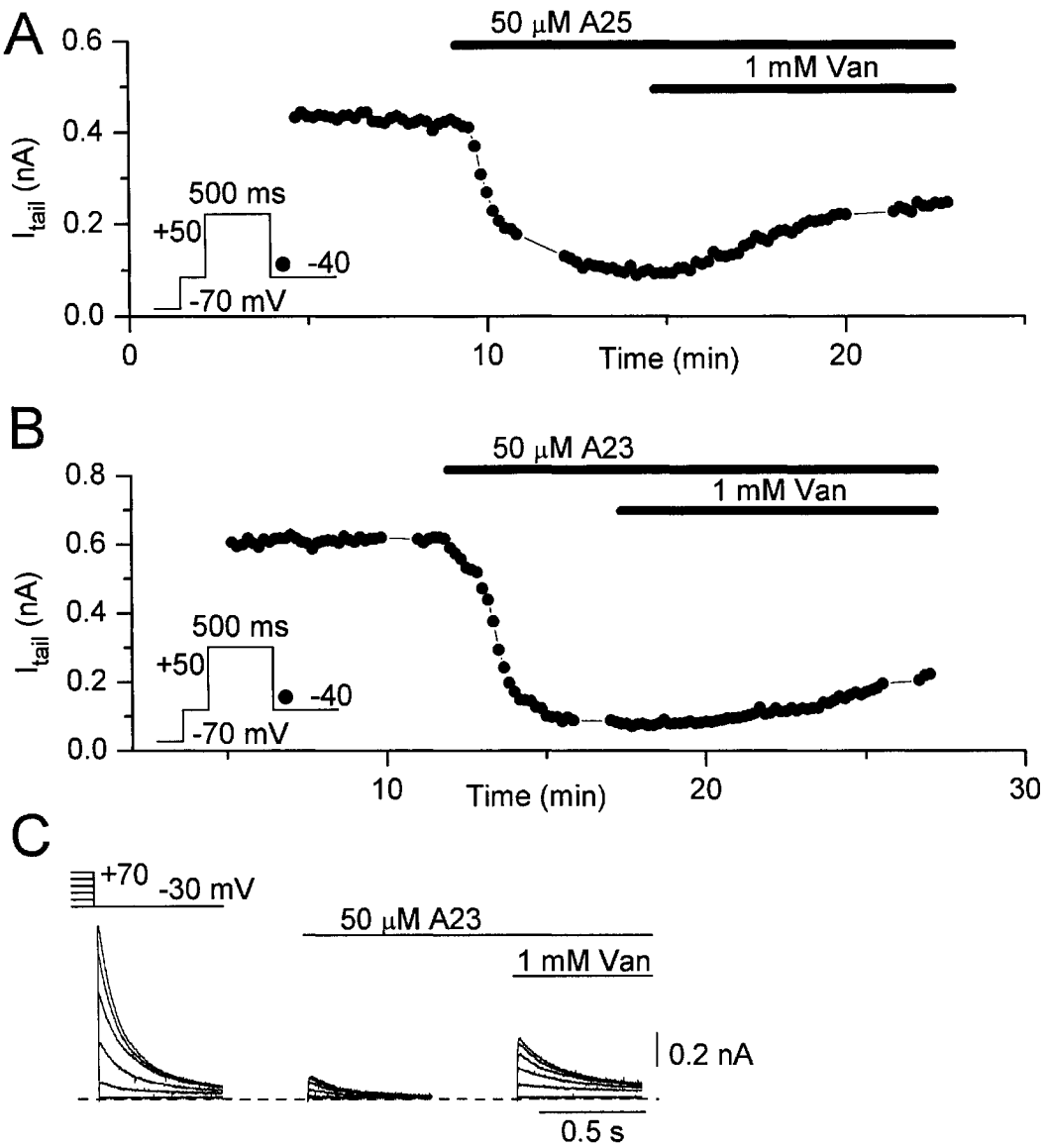

Figure 29: Incomplete restoration of I_{Ks} amplitude by 1mM Van in myocytes pretreated with high concentrations of A25 and A23. (A, B) Time course of changes in the amplitude of tail I_{Ks}. (C) Records of tail currents recorded on I–V runs at the times indicated in B.

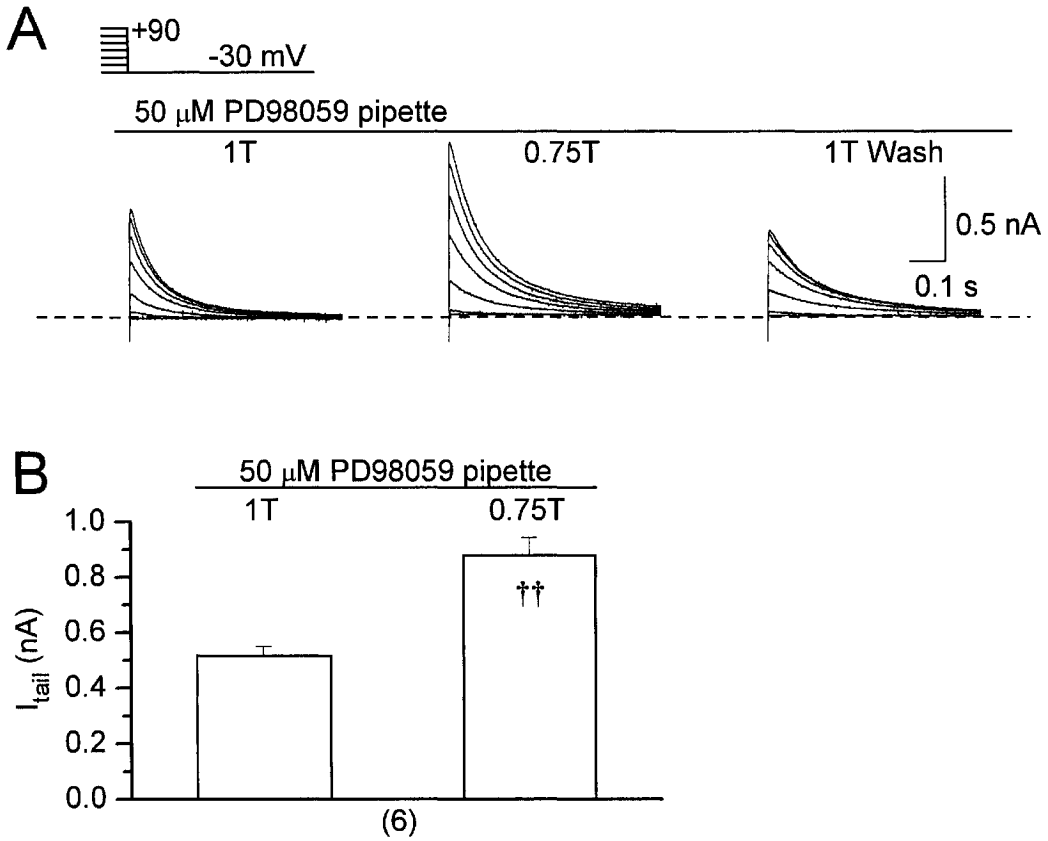

Figure 30: Pretreatment of myocytes with PD98059 does not prevent stimulation of I_{Ks} by 0.75T solution. The cells were pretreated for 2 hours in storage solution that contained 50 μM PD98059-containing solution, and patched with pipette solution that contained 50 μM of PD98059. (A) I_{Ks} tails recorded before, during, and after exposure of pretreated myocyte to hyposmotic solution. (B) Summary of the effects of 0.75T solution on I_{Ks} amplitude in six myocytes pretreated with 50 μM PD98059. †† $p < 0.001$ versus 1T, paired t-test.

3.6 Regulation via MEK and PI3-K

3.6.1 Experiments with PD98059

To investigate whether the stimulatory effects of hyposmotic solution on I_{Ks} were mediated via swelling-induced activation of MAP kinase (ERK1 and 2 subfamilies), experiments were conducted with the MAP kinase kinase (MEK) inhibitor, PD98059 (Alessi *et al.*, 1995). In control experiments, external application of 50 μM PD98059 for 10 min had no effect on the amplitude of I_{Ks} in myocytes bathed in 1T solution (reduction of 4 $\pm$ 4%; n = 4). For the hyposmotic trials, myocytes were pretreated for 2 h in storage solution that contained 50 μM PD98059, a concentration that suppresses activity in the MAPK/ERK cascade with little inhibition of other kinases (Davies *et al.*, 2000). The pretreated myocytes were then patched with pipettes that contained 50 μM PD98059 pipette solution, and exposed to 0.75T solution after equilibration in 1T solution. The drug-treated myocytes had an I_{Ks} density typical of non-treated cells under 1T conditions, and exposure to 0.75T solution increased the amplitude of tail I_{Ks} by 71 $\pm$ 7% (n = 6; $p < 0.001$) (Figure 30). These results suggest that MAP kinase has little regulatory influence on either basal or hyposmotic-stimulated I_{Ks}.

3.6.2 Effects of LY294002

The phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 (Davies *et al.*, 2000) was used to evaluate the possible involvement of PI3-K in the stimulation of I_{Ks} by hyposmotic solution. Myocytes were pretreated with the inhibitor (10 μM) for 8–10 min, and then exposed to hyposmotic LY294002 solution. Even though the

pretreatment decreased the amplitude of I_{Ks} by 54 ± 5% (n = 3), it had no apparent effect on stimulation of the current by hyposmotic solution (increase of 70 ± 14%, n = 3).

3.7 GPCRs, G-proteins, and hyposmotic stimulation of I_{Ks}

There is considerable evidence suggesting that PTKs, including Src, may be functional targets of G-protein coupled receptors (GPCRs) (Chen *et al.*, 1994; Dikic *et al.*, 1996; Diverse-Pierluissi *et al.*, 1997; Felsch *et al.*, 1998). Invariably, the coupling G-protein is of the $G\alpha_o$ or $G\alpha_q$ variety, raising the question whether $G\alpha_q$ in particular might be involved in the pathway that activates I_{Ks} during hyposmotic swelling. In that regard, it has been proposed that stimulation of the $G\alpha_q$-coupled angiotensin II receptor causes activation of PTK in cardiac cells (Sadoshima *et al.*, 1996), and that stimulation of purinergic P2 receptors activates a signaling pathway in cardiac cells that leads to PTK-mediated phosphorylation of proteins, including PLCγ, the anion exchanger AE1 (Puceat and Vassort, 1996; Puceat *et al.*, 1998), and K^+ channels (Matsuura *et al.*, 1996). In addition, it has been suggested that these receptors may play a role in the early responses of smooth muscle (Hamada *et al.*, 1998) and cardiac cells (Sadoshima *et al.*, 1996; Puceat *et al.*, 1998) to mechanical stretch. Cardiac I_{Ks} is inhibited by application of angiotensin II (Daleau and Turgeon, 1994) and stimulated by external ATP (Matsuura and Ehara, 1997; Matsubayashi *et al.*, 1999), and therefore the appropriate test for their involvement in the stimulation of I_{Ks} by hyposmotic solution was to pretreat myocytes with these agonists, and determine whether

this blunted the response of I_{Ks} to subsequent exposure to hyposmotic solution.

Two trials were undertaken with 100 nM angiotensin II, with results (hyposmotic-induced increases of 68 and 77%) similar to those obtained in non-treated myocytes. Five trials were conducted with 20 μM ATP, and records obtained from one of these is shown in Figure 31A. In this pretreated myocyte, the exposure to hyposmotic ATP solution increased I_{Ks} by $\approx$100%; on average, the increase was 72 $\pm$ 15% (n = 4; $p < 0.01$).

A further approach to determining the possible involvement of GPCRs (and G-proteins in general) in the hyposmotic stimulation of I_{Ks} was to dialyze myocytes with a pipette solution that contained a high (10 mM) concentration of inhibitory GDPβS (Eckstein *et al.*, 1979; Gilman, 1987). The GDPβS dialysate had no apparent inhibitory or stimulatory effect on the amplitude of the I_{Ks} tail under 1T conditions, and it failed to inhibit the stimulation of I_{Ks} by hyposmotic 0.75T solution (e.g., Figure 31B). In four myocytes, the hyposmotic solution increased the amplitude of the I_{Ks} tail by 93 $\pm$ 11% ($p < 0.01$). A novel finding in these experiments was that intracellular GDPβS produced a voltage-dependent block of I_{Ks} that resulted in time-dependent currents at higher positive potentials being smaller than tail currents at -30 mV (Figure 31C). The transition from large to small time-dependent current occurred shortly after patch breakthrough, confirming that there was efficient diffusion of GDPβS from the pipette to the membrane.

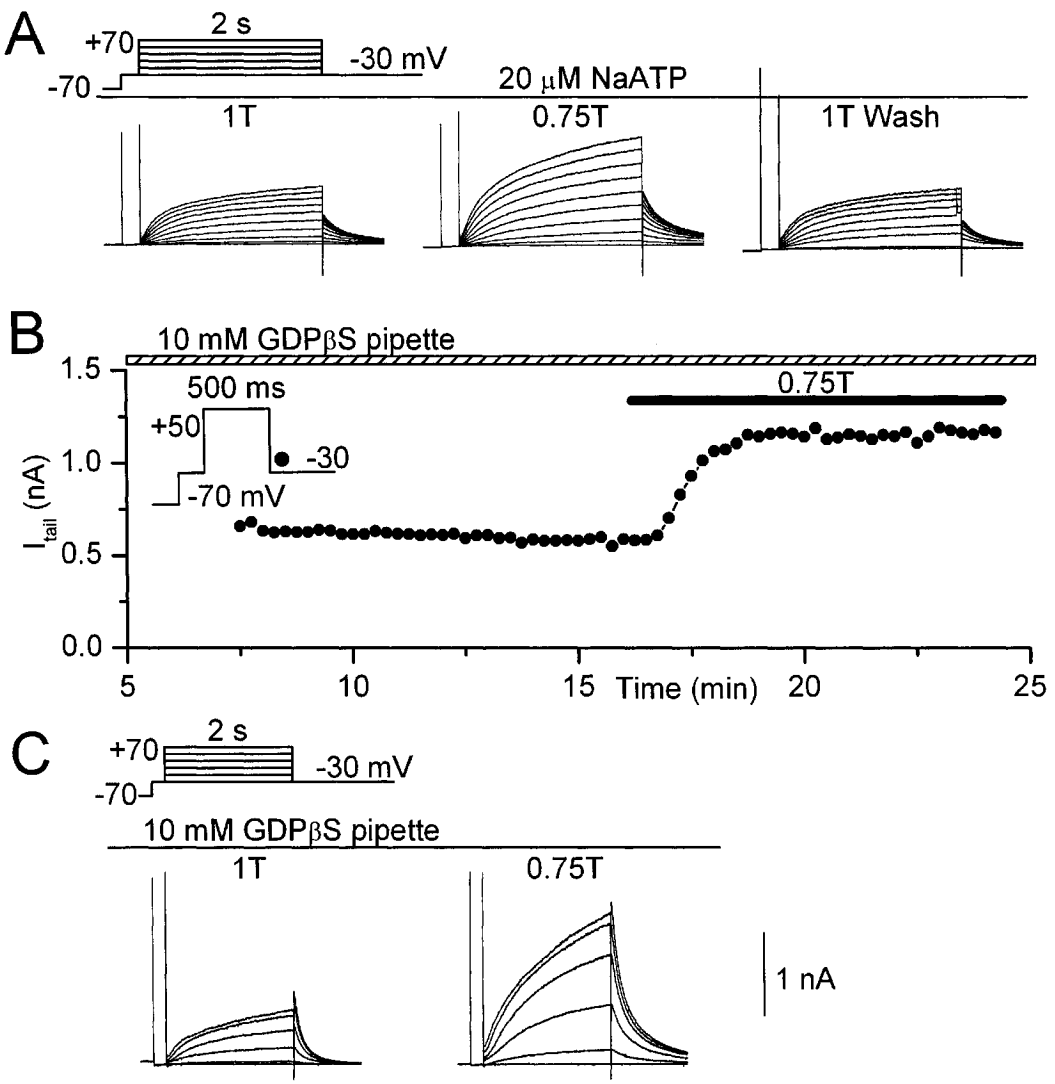

Figure 31: Effects of extracellular ATP and intracellular GDPβS on the stimulation of I_{Ks} by 0.75T solution. (A) Effect of 8-min pretreatment with 20 μM ATP on subsequent stimulation of I_{Ks} by hyposmotic ATP solution. (B, C) Myocytes were patched with pipettes that contained 10-mM GDPβS solution, and exposed to 0.75T external solution at $\approx$10 min post-patch-breakthrough. The GDPβS pretreatment had no apparent effect on the stimulation of I_{Ks} by 0.75T solution in the two representative myocytes. Note the small time-dependent currents relative to the tail currents in the records shown in C.

3.8 Possible involvement of the cytoskeleton

The fungal alkaloid cytochalasin D inhibits the addition of G actin to a nucleation site and thereby disrupts actin-dependent processes in cells (Cooper, 1987). Previous studies have shown a lack of effect of cytochalasin D on the basal activity of KCNQ1/KCNE1 channels expressed in COS-7 cells (Loussouarn *et al.*, 2003), as well as on the stimulation of I_{Ks} by pressure inflation of cardiac myocytes (Wang *et al.*, 1997). On the other hand, incubation with cytochalasin D blocked hyposmotic stimulation of KCNQ1 channel activity in *Xenopus* oocytes (Busch *et al.*, 1992; Grunnet *et al.*, 2003).

Myocytes were pretreated with 10 μM cytochalasin D (CytD) for 10–15 min. This pretreatment, which has been reported to disrupt the cytoskeleton in guinea-pig ventricular myocytes (Pascarel *et al.*, 2001), had no significant effect on tail I_{Ks} amplitude. Subsequent exposure to hyposmotic drug solution resulted in a 77 $\pm$ 10% (n = 4) increase in the tail current amplitude ($p < 0.01$), suggesting that hyposmotic modulation of I_{Ks} does not depend on cytoskeleton organization.

3.9 Modulation of KCNQ1/KCNE1 channel activity by hyposmotic solution and A25

Cardiac I_{Ks} is carried by voltage-gated heteromeric K^+ channels. The product of the KCNQ1 gene (pore-forming domain) coassembles with the product of the KCNE1 gene (auxiliary domain) to form functional Ks channels (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996). The genes can be transiently expressed in a cell line, resulting in functional channels suitable for electrophysiological investigation (Barhanin *et al.*,

1996; Sanguinetti *et al.*, 1996). In the present study, these genes were transiently expressed in BHK cells to determine the response of the KCNQ1/KCNE1 channels to hyposmotic 0.75T solution, A25, and hyposmotic solution in the presence of A25.

Figure 32A, B depicts the effects of hyposmotic solution on KCNQ1/KCNE1 currents in a BHK cell. The hyposmotic solution caused a large (≈3-fold) reversible stimulation of the current. In experiments on cells that were repolarized to −40 mV after 500-ms depolarizations to +50 mV, exposure to hyposmotic solution increased the amplitude of the tail current by 259 ± 38% (n = 8; $p < 0.001$).

The KCNQ1/KCNE1 current was sensitive to A25. In four cells bathed in 1T solution, application of 10 μM A25 for 10 min reduced the amplitude of the tail current to 44 ± 17% of the control value ($p < 0.05$).

To determine whether A25 antagonizes the stimulation of KCNQ1/KCNE1 current by hyposmotic solution, cells were pretreated with 10 μM A25 for 6–8 min, and then exposed to hyposmotic A25 solution. The tail current records obtained from one of these cells indicate that the A25 pretreatment did not prevent stimulation by hyposmotic solution (Figure 32C). However, the degree of stimulation was blunted by the PTK inhibitor; compared to the 259 ± 38% increase in tail current amplitude observed in control cells, the increase in A25-pretreated cells was a significantly ($p < 0.05$) smaller 92 ± 22% (n = 4) (Figure 32D). In summary, KCNQ1/KCNE1 current in BHK cells responded to hyposmotic stimulation and A25 in a manner that was qualitatively similar to that of I_{Ks} in cardiac myocytes.

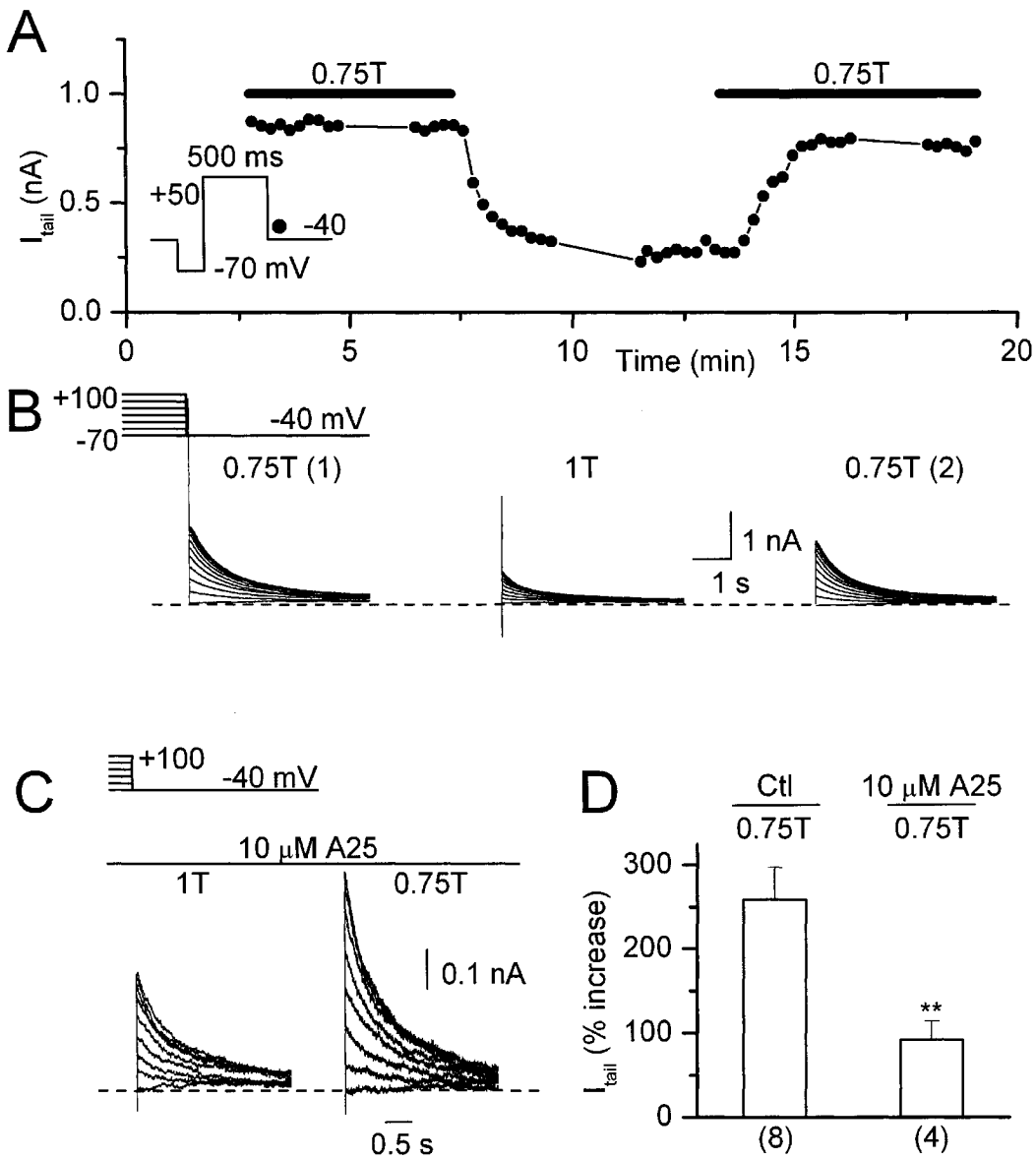

Figure 32: Response of KCNQ1/KCNE1 current to hyposmotic solution and A25. (A, B) Stimulation of current in a BHK cell exposed to 0.75T solution. (C) Records obtained from a representative cell that was pretreated with 10 μM A25 for 6–8 min and then exposed to hyposmotic solution. (D) Summary of results obtained from control and A25-pretreated myocytes. ** $p < 0.05$ versus response of control cells, unpaired t-test. Numbers of cells in parentheses.

4

Discussion

The discussion is divided into six sections. The first section (4.1) discusses the effects of hyposmotic solution on the properties of cardiac I_{Ks}. The second section (4.2) describes the possible effects of I_{Ks} stimulation on the properties of the cardiac action potential and cardiac myocyte repolarization. Sections 4.3–4.5 consider the role of serine/threonine kinase, MAPK, PI3-K, G-proteins, and the cytoskeleton in stimulation of I_{Ks} by hyposmotic solution. In section 4.6 the role of tyrosine phosphorylation in I_{Ks} stimulation under hyposmotic conditions is discussed.

4.1 Effects of hyposmotic solution on I_{Ks} and KCNQ1/KCNE1 current

Sasaki *et al.* (1992, 1994) were the first to show that extracellular hyposmotic solution stimulates I_{Ks} in guinea pig ventricular myocytes. Since then, there have been further reports on hyposmotic stimulation of I_{Ks} in guinea pig (Rees *et al.*, 1995; Groh *et al.*,

1996; Kocic *et al.*, 2001; Ogura *et al.*, 2003) and canine (Zhou *et al.*, 1997) ventricular myocytes. There has also been one study on the hyposmotic stimulation of currents carried by KCNQ1 and KCNQ1/KCNE1 channels expressed in COS-7 cells (Kubota *et al.*, 2002), and one on KCNQ1/KCNE1 channels expressed in *Xenopus* oocytes (Grunnet *et al.*, 2003).

4.1.1 Effects on I_{Ks} amplitude and kinetics

In the present study, 0.75T hyposmotic solution increased the amplitude of tail I_{Ks} by an average of 73%. This value is in good agreement with the 78% increase (0.7T solution) reported by Sasaki *et al.* (1994), but somewhat larger than the 46% increase (0.67T solution) reported by Rees *et al.* (1995), and the 60% increase (0.6T solution) reported by Zhou *et al.* (1997).

The amplitude of I_{Ks} increased within 1–3 min from the onset of cell superfusion by 0.75T solution, and reached steady state within 3–5 min. This time course of the current stimulation by hyposmotic solution is in agreement with that previously described for hyposmotic (0.6T) stimulation of I_{Ks} in guinea pig ventricular myocytes (Kocic *et al.*, 2001). In canine ventricular myocytes studied at 25–27°C, the onset of stimulation by 0.6T solution occurred within 5 min, and the stimulation reached steady state in 10–12 min (Zhou *et al.*, 1997). The increase of I_{Ks} amplitude on myocyte swelling was slower than the decrease of I_{Ks} amplitude on myocyte shrinkage (see Figure 6B). In that regard, the half-time of rabbit ventricular myocyte swelling in 0.5T solution was significantly slower than the half-time of myocyte shrinkage upon switching back to 1T conditions (37 s versus 25 s) (Suleymanian and Baumgarten, 1996). A similar pattern of swelling/shrinkage kinetics exists in guinea pig ventricular

myocytes (Ogura *et al.*, 2002) and may be a reason for the apparent difference in I_{Ks} amplitude time course between myocyte swelling and shrinkage.

The average value of the $V_{0.5}$ of I_{Ks} activation under control conditions (+19 ± 0.6 mV) in the present study is similar to the $V_{0.5}$ values (+15.7, +23.7, and +18.7 mV) obtained in earlier studies on guinea pig ventricular myocytes by Sanguinetti and Jurkiewicz (1990), Fan and Hiraoka (1991), and Ogura *et al.* (2003), respectively. The slope of the approximated activation relationship found here (+15 ± 0.4 mV) is also in good agreement with earlier values (+12.7, +19.6, and +13 mV) reported by Sanguinetti and Jurkiewicz (1990), Fan and Hiraoka (1991), and Matsubayashi *et al.* (1999), respectively.

In the present study, exposure of myocytes to hyposmotic 0.6T solution caused an insignificant 2 mV leftward shift in the $V_{0.5}$ of the approximated activation relationship of I_{Ks}. This finding is in good agreement with earlier reports on the effects of hyposmotic solution on $V_{0.5}$ in these myocytes (Groh *et al.*, 1996; Kocic *et al.*, 2001; Ogura *et al.*, 2003).

To determine the kinetics of I_{Ks} deactivation, tail currents elicited by repolarization to −30 mV after 2-s depolarizations to +50 mV were fitted with single exponential functions. Under isosmotic conditions, the time constant of deactivation was 165 ± 17 ms. This value is in reasonable agreement with the values obtained in earlier studies on I_{Ks} in canine ventricular myocytes (Liu and Antzelevitch, 1995) (130 ms), human ventricular myocytes (122 ms) (Virag *et al.*, 2001), and guinea pig ventricular myocytes (Heath and Terrar, 1996b) (257 ms).

To determine the effects of hyposmotic solution on the deactivation kinetics, tail currents elicited after 500-ms depolarizations to +50 mV were fitted with double

exponential functions. Measurements were taken just before the application of 0.75T solution, shortly after current amplitude had reached a new steady state, and again after washout of the response. There was no significant change in the deactivation time constant at these times. The average value of the fast time constant was 97 ms, and the average value of the slow time constant was 363 ms. These values are considerably smaller than the fast (203 and 169 ms) and slow (741 ms and 1.24 s) time constants found in earlier studies on I_{Ks} in guinea pig (Lu *et al.*, 2001) and canine (Liu and Antzelevitch, 1995) ventricular myocytes, respectively. A possible cause for the discrepancy is that the length of the activating pulse was 500 ms in the present study, versus 3 s in the study of Lu *et al.* (2001), and 5 s in in the study of Liu and Antzelevitch (1995). However, smaller values were obtained in one study on guinea pig ventricular myocytes: fast constant of 216 ms and slow constant of 479 ms (currents were elicited by 3-s pulses) (Bosch *et al.*, 1998).

To determine the effects of hyposmotic solution on the activation kinetics of I_{Ks}, the current elicited by 2-s pulses to +50 mV was fitted with double exponential functions. There were no significant changes in the fast (122 $\pm$ 22 ms, versus 132 $\pm$ 36 ms) and slow (1125 $\pm$ 155 ms, versus 1237 $\pm$ 130 ms) time constants under isosmotic and hyposmotic conditions, respectively. These results indicate that the increase in I_{Ks} amplitude induced by hyposmotic solution was not caused by an acceleration of current activation.

4.1.2 Effects on KCNQ1/KCNE1 current

There have been two earlier studies on the effects of hyposmotic solution on expressed KCNQ1 and KCNQ1/KCNE1 channels. Application of ≈0.7T solution produced ≈100% increases in the amplitudes of KCNQ1 and KCNQ1/KCNE1 currents in COS-7 cells (Kubota *et al.*, 2002), and a 72% increase in the amplitude of KCNQ1 current in *Xenopus* oocytes coexpressing aquaporin AQP1 channels (Grunnet *et al.*, 2003). In these two studies, the exposures to hyposmotic solution caused little change in the voltage dependencies of current activation. The fact that KCNQ1 is stimulated by hyposmotic solution (Kubota *et al.*, 2002; Grunnet *et al.*, 2003) provides a strong indication that KCNQ1 is the osmosensitive subunit in the KCNQ1/KCNE1 protein complex. Whether and how KCNE1 might affect the response of KCNQ1 to hyposmotic solution is unclear. Kubota *et al.* (2002) reported that KCNQ1/KCNE1 current was stimulated to the same degree as KCNQ1 current, whereas Grunnet *et al.* (2003) found that KCNQ1/KCNE1 current was more strongly stimulated. On the other hand, Lock and Valverde (2000) found that epithelial cells from KCNE1 knockout mice had greatly diminished regulatory volume decrease responses, suggesting a stimulatory role for KCNE1 in normal epithelial cells.

In the present study, experiments were performed on BHK cells transiently expressing KCNQ1/KCNE1 channels. Application of 0.75T hyposmotic solution caused a large (259%) average increase in the amplitude of KCNQ1/KCNE1 current. This degree of stimulation of the current is significantly larger than the ≈73% increase obtained in guinea pig cardiac myocytes superfused with 0.75T solution, and larger than the ≈100% increase in the amplitude of KCNQ1/KCNE1 current in COS-7 cells (Kubota *et al.*, 2002). The difference in response between the native and expressed

channels could be related to the difference in cell type (cardiac myocytes versus BHK cells), whereas that between BHK and COS-7 cells may be related to differences in activity of cell signaling mechanisms in the two cell lines.

4.1.3 Possible mechanisms underlying the stimulation of I_{Ks}

The mechanism(s) responsible for the stimulation of cardiac I_{Ks} by hyposmotic external solution have not yet been clarified. Mechanisms that might play a role include: (i) mechanical deformation or stretch of the cell membrane, (ii) reorganization of the cytoskeleton and activation of cytoskeleton-related signaling, (iii) change in channel trafficking, (iv) change in channel protein hydration state, (v) change in protein–lipid interactions in the membrane, (vi) change in the interaction of channels with other proteins, (vii) change in the phosphorylation status of channels, (viii) macromolecular crowding, and (ix) change in cytoplasmic ion concentrations. Recent reviews on the overall subject include those by Baumgarten and Feher (2001), Hamill and Martinac (2001), and Poolman *et al.* (2002).

Cell membrane deformation. Cell membrane deformation in response to hyposmotic stress can be characterized by a thinning of the membrane and an increase in membrane area (Hamill and Martinac, 2001). One can speculate that such a decrease in membrane thickness could lead to a change in Ks channel–membrane lipid interaction, and thereby activate channels. For an "unfolded" membrane, there is a linear relationship between the increase in membrane tension, the relative membrane area, and the relative membrane thickness (Evans and Hochmuth, 1978). However, as shown in Figure 7, there is a sigmoidal relationship between the inverse of extracellular osmolarity and relative change in I_{Ks} amplitude. Therefore, it is unlikely that

there is a direct correlation between cell membrane deformation and I_{Ks} amplitude under hyposmotic conditions.

The completely unfolded plasma membrane in animal cells can stretch only by about 2–3% before it ruptures (Nichol and Hutter, 1996; Morris and Homann, 2001). Therefore, a relatively large increase in cell volume upon hyposmotic swelling can be achieved either by unfolding of membrane invaginations or microvilli (Sukhorukov *et al.*, 1993; Raucher and Sheetz, 1999), or by an increase in exocytosis and addition of new membrane (discussed below). In case of the former, the loss of membrane invaginations and membrane deformation can lead to the stimulation of mechanosensitive ion channels Suchyna *et al.* (2000); Vanoye and Reuss (1999) and rapid reorganization of the cytoskeleton (Morris and Homann, 2001; Sun and Levitan, 2003) (also see below).

Cytoskeleton reorganization. Hyposmotic-induced stress-related cytoskeleton reorganization and modified signaling can affect the properties of ion channels. For example, hyposmotic stimulation of Ca^{2+}-activated K^+ channels expressed in *Xenopus* oocytes was abolished by 30-min pretreatment with cytoskeleton-disrupting cytochalasin D (1 μM) (Grunnet *et al.*, 2002). The possible involvement of the cytoskeleton in stimulation of cardiac I_{Ks} by hyposmotic solution is discussed in a separate section below.

Change in channel trafficking. Activation of $I_{Cl,\,swell}$ in rabbit nonpigmented ciliary epithelial cells by hyposmotic solution has been associated with an increase in channel subunit trafficking to the cell membrane that occurs within 5–7 min from the application of the solution (Vessey *et al.*, 2004). Stimulation of Na^+ current

conducted by hH1 Na^+ channel subunits expressed in *Xenopus* oocytes occurred with a lag of 5–10 min when channel trafficking was stimulated by a stimulation of PKA (Zhou *et al.*, 2000). A slow increase in vesicular transport (20% increase in surface fluorescence after 6 min hyperosmotic challenge) and associated slow increase in nonselective cation (NSC) current has also been reported for HTC cells (Feranchak *et al.*, 2003).

Channel trafficking to the cell membrane is associated with exocytosis and insertion of new membrane (which increases cell membrane capacitance (C_m)). However, it is worth noting that addition of new membrane during hyposmotic swelling was ruled out in a study on cultured chick cardiac myocytes because the swelling and increase in the amplitude of $I_{\mathrm{Cl,\,swell}}$ was not accompanied by a change in C_m (Zhang *et al.*, 1997). Similarly, swelling-induced increase in rat hepatocyte membrane conductance was not accompanied by an increase in C_m (Graf *et al.*, 1995). In contrast to the foregoing, extreme osmotic swelling (bathing with distilled water) caused a reversible increase in the membrane capacitance of snail neurons (Wan *et al.*, 1995). A significant increase in C_m has also been detected in HT_{29} cells within 2–3 min of application of 160 mosm external solution (Greger *et al.*, 1993), and an increase in C_m was detected within 30 s of hyposmotic challenge in human epithelial cells (Okada *et al.*, 1992).

Part of the t-tubular system may represent a membrane reservoir that fuses with outer membrane under hyposmotic conditions and contributes additional Ks channels. However, detubulation of cardiac myocytes with formamide leads to a *decrease* in C_m and decrease in Ca^{2+} current amplitude (Kawai *et al.*, 1999). In addition, detubulation did not increase delayed rectifier potassim current amplitude, suggesting that

formamide-induced osmotic shock does not increase the number of active channels. Hyposmotic shock induced by 0.6T solution did not cause a loss of t-tubule striation pattern in rat ventricular myocytes, indicating that moderate swelling does not affect transverse tubules (Brette *et al.*, 2000).

In the present study, C_m was not monitored and therefore it is not possible to state whether during application of hyposmotic solution there was an addition of cell membrane that contained Ks channels. However, myocyte I_{Ks} responded to the application of hyposmotic solution much faster (within 1–3 min) than the trafficking of ion channel subunits in the studies by Zhou *et al.* (2000) and Feranchak *et al.* (2003), suggesting that enhanced trafficking is not the key mechanism responsible for hyposmotic stimulation of I_{Ks}.

Altered protein hydration. A change in the hydration state of Ks channel protein is another possible mechanism that may be involved in the stimulation of I_{Ks} by hyposmotic solution. Zimmerberg *et al.* (1990) have suggested that activation of voltage-gated K^+ channels in squid axon depends on both voltage- and osmotically-dependent gating steps. The extra work required to move water into the solute-inaccessible region of the K^+ channel pore under *hyperosmotic* conditions determines the lower open probability of the channels in shrunken axons. It should be noted, however, that according to the model proposed by Zimmerberg *et al.* (1990), there is a linear relationship between the change in osmotic pressure and channel conductance. The non-linear relationship between the inverse of extracellular solution osmolarity and the relative change in I_{Ks} amplitude obtained in the present study suggests that, at the very least, other mechanisms are involved in the modulation of I_{Ks} by anisosmotic solution. In addition, I_{Ks} can be stimulated by mechanical inflation of

cardiac myocytes (Wang *et al.*, 1996), suggesting that lowering of solution osmolarity *per se* is not a mechanism of the current stimulation.

Altered membrane protein–lipid interaction. The degree of membrane bending or change in curvature under hyposmotic conditions depends on the membrane lipid composition. Above the phase transition temperature, the lipid bilayer behaves as a fluid with negligible rigidity to mechanical stress (Hamill and Martinac, 2001). However, heterogeneity of membrane content, such as the presence of regions rich in sphingolipids and cholesterol (membrane rafts) (Simons and Ikonen, 1997) can create areas with different mechanical properties and responses to mechanical stress (Hamill and Martinac, 2001). Certain expressed and native voltage-gated K^+ channels (e.g., Kv2.1 family) target to lipid rafts (Martens *et al.*, 2000). Despite the fact that no such targeting has been reported for native Ks channels or channels of the KCNQ family, the possibility exists that these channels can associate with specific membrane microdomains and form an osmosensitive complex.

Altered channel phosphorylation status. A classical example of an osmosensitive signaling molecule is the membrane-bound histidine kinase KdpD bacterial sensor. The activity of this kinase increases with an increase in the ionic strength of the medium. Under hyperosmotic conditions, KdpD activates response regulator KdpE, and induces expression of the kdpFABC operon, which in turn increases K^+ uptake (Sugiura *et al.*, 1994). Similar two-component osmosensing/osmoregulation mechanism in bacterial cells also involves EnvZ (the sensor) and OmpR (the regulator). At high osmotic pressure, EnvZ autophosphorylates on the histidine residue, activates OmpR, which in turn increases the expression of outer membrane porines

(Forst *et al.*, 1989). A yeast histidine kinase Sln1p is a likely candidate for the eukaryotic osmosensor, and its activity is mediated by the HOG (Hog1) pathway (Hohmann, 2002). Hog1 is homologous to the mammalian p38/JNK MAPK kinase, and, perhaps not surprisingly, MAPK (ERK, p38, and JNK) pathways are stimulated by hyposmotic stress (Tilly *et al.*, 1996; Sadoshima *et al.*, 1996; Niisato *et al.*, 1999; Obata *et al.*, 2000). The involvement of kinases and phosphatases in the stimulation of cardiac I_{Ks} by hyposmotic solution is discussed at a later point below.

Macromolecular crowding. A small change in the concentration of inert macromolecules can greatly change the properties of cell proteins (Minton, 1997), including enzymes (up to several thousand fold change in activity). This phenomenon is termed "macromolecular crowding", and is related to the interaction of cell proteins with the local macromolecular environment (for review, see Al-Habori, 2001). Therefore, macromolecule concentration can act as a cellular osmotic sensor and directly or indirectly (by modulating the activities of cell signaling pathways) affect the activity of ion channels. The present results provide no information on whether changes in macromolecular crowding was involved in the stimulation of I_{Ks} by hyposmotic solution. Possible modulation of kinase/phosphatase activity by macromolecular crowding is discussed at a later point below.

Intracellular ion concentration. When myocytes are exposed to hyposmotic solution, there is a reduction in the concentrations of ions in the cytoplasm due to expansion of cytoplasmic water (Lei and Kohl, 1998). Since the intracellular concentrations of Mg^{2+} ($[Mg^{2+}]_i$), Ca^{2+} ($[Ca^{2+}]_i$), H^+ ($[H^+]_i$), and K^+ ($[K^+]_i$) can affect cardiac I_{Ks}, the possibility that changes in their concentrations underlie the effects

of hyposmotic solution on the current needs to be considered.

A decrease in $[Mg^{2+}]_i$ might lead to an increase in the amplitude of I_{Ks} because $[Mg^{2+}]_i$ is an endogenous blocker of Ks channels (Williams and Beatch, 1997). However, there are several reasons why this is unlikely to be the mechanism responsible for the stimulation of I_{Ks} by hyposmotic solution. First, the calculated free Mg^{2+} in the pipette solutions used in the present study was 0.89 mM, a value that is 45.000 times higher than the EC_{50} of 24 nM estimated for Mg^{2+} block of I_{Ks} in guinea pig ventricular myocytes by Williams and Beatch (1997). Therefore, after the relatively small reduction of $[Mg^{2+}]_i$ that might occur under hyposmotic conditions, I_{Ks} is still largely blocked by $[Mg^{2+}]_i$, and the current amplitude is unlikely to change as a result. Second, a decrease in $[Mg^{2+}]_i$ from 87 μM to 100 nM shifted the activation relationship of I_{Ks} by -17 mV (Williams and Beatch, 1997), whereas hyposmotic solution had no effect on $V_{0.5}$ in the present study. Third, in a study by Hirahara *et al.* (1998) when $[Mg^{2+}]_i$ was in the range 0.03 to 1 mM, I_{Ks} amplitude in guinea pig ventricular myocytes was relatively stable, with marked rundowns occurring with $[Mg^{2+}]_i$ above 3 mM and below 0.01 mM. In summary, dilution of $[Mg^{2+}]_i$ is unlikely to be the reason for the stimulation of I_{Ks} by hyposmotic solution.

Tohse (1990) has reported that a lowering of $[Ca^{2+}]_i$ below 1 nM had no effect on I_{Ks} in guinea pig ventricular myocytes, whereas an elevation from 10 to 100 nM increased the amplitude of the current by $\approx$3-fold. Nitta *et al.* (1994) reported that there was an $\approx$60% increase in the amplitude of I_{Ks} in inside-out patches excised from guinea pig ventricular myocytes when $[Ca^{2+}]_i$ was increased from 10 nM to 1 μM. In the present study, $[Ca^{2+}]_i$ was buffered to $<$1 nM with EGTA, and therefore a further lowering of concentration due to cell swelling is unlikely to have been of much

consequence. Additionally, Boucherot *et al.* (2001) have found that KCNQ1 requires coassembly with KCNE1 or KCNE3 in order to gain sensitivity to $[Ca^{2+}]_i$, suggesting that the hyposmotic stimulations of homomeric KCNQ1 current reported by Kubota *et al.* (2002) and Grunnet *et al.* (2003) were not related to changes in $[Ca^{2+}]_i$.

There is no information available on the effects of changes in intracellular pH (pH_i) on I_{Ks}. However, it has been reported that cytoplasmic acidification causes a stimulation of KCNQ1/KCNE1 current (Unsold *et al.*, 2000). If that is the case for I_{Ks}, dilution of myocyte $[H^+]_i$ during hyposmotic swelling might lead to an inhibition of I_{Ks}, rather than a stimulation observed here.

Application of hyposmotic solution is expected to lower $[K^+]_i$ and cause a lowering of the driving force for outward K^+ current at a given membrane potential. Such a lowering cannot explain the increase in I_{Ks} amplitude observed under hyposmotic conditions.

4.2 Hyposmotic stimulation of I_{Ks} affects cardiac myocyte repolarization

4.2.1 Effects of I_{Ks} augmentation on the action potential duration

The increase in I_{Ks} amplitude under hyposmotic conditions increases the net outward current during the repolarization phase of the cardiac action potential (Bosch *et al.*, 1998). An increase of this magnitude causes a shortening of the action potential duration (APD) in cardiac ventricular myocytes (Sanguinetti and Jurkiewicz, 1990).

The effect of increased I_{Ks} on the APD has been demonstrated in the present study (Figure 8) using a computer model of cardiac action potential. According to the calculated data, an increase in I_{Ks} amplitude of the magnitude elicited by application of 0.75T solution decreases APD_{90} from basal 160 ms to 136 ms at a basic cycle length of 800 ms. However, the role of I_{Ks} in the action potential repolarization of real myocytes depends on the species: APD in canine (Varro *et al.*, 2000) and rabbit (Lengyel *et al.*, 2001) ventricular myocytes was little affected by pharmacological block of I_{Ks} with chromanol 293B (10 μM) and L-735,821 (100 nM), whereas human and guinea pig action potentials were prolonged by the application of I_{Ks} blocker chromanol 293B (1 μM) (Bosch *et al.*, 1998) and APD in dog ventricular myocytes treated with isoproterenol was increased by chromanol 293B (30 μM) and HMR 1556 (500 nM) (Volders *et al.*, 2003).

The computed APD shortening under hyposmotic conditions is in agreement with results obtained in earlier studies on guinea pig ventricular myocytes. Application of 0.7T solution shortened APD_{90} from 180 (control) to 132 ms (Kocic *et al.*, 2001) and application of 0.6T solution decreased APD_{90} from 171 (control) to 132 ms (Kocic *et al.*, 2004). In myocytes pretreated with 10 μM E4031 at room temperature, application of hyposmotic solution shortened the APD_{90} from 810 ms to 510 ms (37% decrease) (Groh *et al.*, 1996). Taken together, the results suggest that hyposmotic-induced increase in I_{Ks} amplitude causes a significant decrease in APD in guinea pig ventricular myocytes.

APD prolongation under basal pacing rate ultimately leads to an increase in the QT interval. Lengthening of QT interval may have both antiarrhythmic and proarrhythmic effects. The antiarrhytmic effect of APD prolongation is related to the

lengthening of the refractory period and abolishment of reentry, whereas the proarrhythmogenic effect arises from the increased likelihood of afterdepolarizations and from an increase in the repolarization heterogeneity (January *et al.*, 1991). Mutations in the KCNQ1 and KCNE1 proteins, and pharmacological block of I_{Ks}, cause a prolongation of QT interval and lead to the form of long QT syndrome termed LQT1 (Wang *et al.*, 1996; Chen *et al.*, 1999).

The increase in I_{Ks} under hyposmotic conditions, and resultant shortening of the APD, can therefore be seen as a compensatory factor under conditions of prolonged QT interval. However, under conditions of severe ventricular arrhythmia, class III antiarrhythmic compounds are often prescribed (Anderson and Prystowsky, 1999; Igawa *et al.*, 2002). These drugs work by blocking cardiac delayed rectifier currents, thereby *lengthening* the APD and, as described above, suppressing reentry (Ward and Gill, 1997; Kodama *et al.*, 1999). Shortening of the APD under hyposmotic conditions decreased the efficiency of the class III antiarrhythmic drug, E-4031 (Groh *et al.*, 1996), and can therefore be considered a risk factor for patients taking class III compounds.

4.2.2 Role of stimulated I_{Ks} in the shaping of the APD restitution curve

The APD restitution curve is an important indicator of the probability of ventricular arrhythmia (Franz, 2003). The APD depends on the time passed since the completion of the previous action potential (i.e., diastolic interval (DI)). The relationship between DI and APD (termed "restitution curve") has an asymptotic behavior: the APD values change rapidly when DIs are relatively short, and approach steady state at

longer DIs. Activation of K^+ currents including I_{Ks} is one of the major factors that shapes the APD restitution curve (Zeng *et al.*, 1995). In the present study, the APD restitution curve was obtained from a computer simulation of guinea pig ventricular action potentials. It exhibits a classical fast decrease in APD_{90} values at short DIs, and a slow asymptotic increase towards a steady APD_{90} value at long DIs. The maximal I_{Ks} conductance in the model was increased by 73% in order to simulate application of 0.75T solution to a myocyte. This increase had two major effects on the APD restitution curve: (i) the slope of the curve at short DI values was shallower, and (ii) the steady state level of the curve at large DI values was lower (reflecting the fact that APD_{90} is smaller when I_{Ks} amplitude is larger). The APD restitution curve slope is of a special importance: steep restitution curves are generally associated with a transition from ventricular tachycardia to ventricular fibrillation (Panfilov, 1998; Riccio *et al.*, 1999). Therefore, the "flattening" of the slope upon hyposmotic stimulation of I_{Ks} may be an indicator of a beneficial antiarrhythmogenic effect of stimulated I_{Ks}.

4.3 Apparent lack of involvement of serine/threonine kinases in the hyposmotic stimulation of I_{Ks}

4.3.1 Effects of PKA modulators on basal and hyposmotically-stimulated I_{Ks}

PKA is a positive regulator of I_{Ks} in cardiac myocytes (Walsh and Kass, 1988; Harvey and Hume, 1989; Yazawa and Kameyama, 1990). To study the effects of PKA on I_{Ks}

under isosmotic and hyposmotic conditions, the following approaches were used: (i) PKA was inhibited by using serine/threonine kinase inhibitors H8 and H89, and (ii) PKA was stimulated by using FSK.

Isosmotic conditions. Dialysis of myocytes with 200-μM H8 pipette solution effectively antagonized the stimulation of I_{Ks} by ISO, indicating strong inhibition of PKA in H8-treated myocytes. In the case of H89, application of 10–100 μM drug extracellularly or 100 μM drug intracellularly had little effect on the amplitude of basal I_{Ks}. The two serine/threonine kinase inhibitors differed in their effects on basal I_{Ks}: H8 inhibited basal current by 50%, whereas H89 had little effect. The reason for this discrepancy may be due to non-PKA-related effects of the drugs, i.e., H89 (10 μM) inhibits PKG, ROCK-II, MSK, and S6K1 (Davies *et al.*, 2000), and H8 (10 μM) is a potent inhibitor of PKG (Kwan *et al.*, 2004) and potentially has a direct blocking action on K^+ channels (Hockberger *et al.*, 1989). The lack of effect of H89 on basal I_{Ks} suggests that basal PKA activity in guinea pig ventricular myocytes pertinent to I_{Ks} was relatively low. An analogous finding with H89 on basal $I_{Ca,\,L}$ in ferret ventricular myocytes (i.e., lack of effect of the inhibitor on basal current), led to a similar conclusion with regard to basal PKA phosphorylation of L-type Ca^{2+} channels in those myocytes (Yuan and Bers, 1995).

Hyposmotic conditions. H8 had little or no effect on the stimulation of I_{Ks} by hyposmotic solution. This result is in agreement with an earlier study on canine ventricular myocytes, in which dialysis with 100 μM H8 solution had no effect on hyposmotic stimulation of I_{Ks} (Zhou *et al.*, 1997). In agreement with the H8 result, neither intracellular nor extracellular application of H89 (10–100 μM) affected the

stimulation of I_{Ks} by hyposmotic solution. The latter result confirms and extends an earlier finding on the lack of effect of 10-μM H89 pipette solution on hyposmotic stimulation of I_{Ks} in guinea pig ventricular myocytes (Groh *et al.*, 1996). Two other results that argue against involvement of PKA in the stimulation of I_{Ks} by hyposmotic solution were that pre-activation of the kinase by application of 1 or 5 μM FSK did not occlude normal hyposmotic stimulation of I_{Ks}, and that hyposmotic stimulation of the current was characterized by an unchanged activation relationship (Figure 10) (whereas PKA-mediated stimulation of I_{Ks} causes a -20 mV shift in the activation relationship of I_{Ks} (Walsh and Kass, 1991; Lo and Numann, 1998)).

4.3.2 Lack of involvement of PKC in the stimulation of I_{Ks} by hyposmotic solution

Previous studies have shown that activation of PKC has diverse effects on swelling-activated currents. For example, volume-sensitive I_{Cl} in human cervical cancer HT-3 cells is downregulated by PKC inhibitors staurosporine and H7 (Chou *et al.*, 1998), and volume-sensitive I_{Cl} in canine atrial cells is stimulated by PKC activator beta-phorbol 12, 13-dibutyrate (PDBu) (Du and Sorota, 1999); conversely, activation of PKC by PDBu and PMA inhibited swelling-activated I_{Cl} in mouse renal inner medullary collecting duct cell line (mIMCD-K) (Boese *et al.*, 2000), NIH/3T3 cells (Duan *et al.*, 1999), and rabbit nonpigmented ciliary epithelial cells (Shi *et al.*, 2002).

Since I_{Ks} in guinea pig ventricular myocytes is stimulated by activation of PKC (Walsh and Kass, 1988; Tohse *et al.*, 1990), it seemed possible that hyposmotic stimulation of I_{Ks} was mediated by activation of the kinase. The involvement of PKC in the hyposmotic stimulation of I_{Ks} was tested by application of (i) PKC inhibitors

H7 and Bis, and (ii) PKC agonist PMA. Even though H7 is widely-used as a PKC inhibitor (Hidaka and Kobayashi, 1992), some of its effects may be unrelated to an inhibition of PKC (Schilling and Eder, 2003). In the present study, H7 decreased the amplitude of I_{Ks} under isosmotic conditions, and prevented PMA-mediated stimulation of the current, whereas structurally-different Bis had no effect on the amplitude of basal I_{Ks}. The inhibitory effect of H7 on basal I_{Ks} may have been due to a direct block of Ks channels or to inhibition of an unidentified kinase by H7 (Hockberger *et al.*, 1989).

H7, Bis, and PMA failed to affect the stimulation of I_{Ks} by 0.75T solution, suggesting that the stimulation is not mediated by activation of PKC. These results extend the findings of Groh *et al.* (1996) who indicated that pretreatment of guinea pig ventricular myocytes with PKC inhibitor chelerythrine (10 μM) had little effect on the stimulation of I_{Ks} by hyposmotic solution. In regard to PMA, it has been reported that stimulation of PKC causes an increase in the slope of the I_{Ks} activation relationship (Walsh and Kass, 1991), whereas application of hyposmotic solution in the present study had no significant effect on the slope. In summary, the results suggest that PKC is not involved in the modulation of I_{Ks} by hyposmotic solution.

4.4 Lack of involvement of MAPK (ERK1/2) in the modulation of I_{Ks} by hyposmotic solution

MAP kinases (MAPKs) are involved in the regulation of a number of ion channels and pumps. In addition, there is a growing evidence on the involvement of ERK and p38 kinase in cellular responses to mechanical and osmotic stress. In cultured human

umbilical vein endothelial cells, shear mechanical stress produced stimulation of MAP kinase (preferentially ERK2) within 5 min, with a peak at 15 min (at 37 °C) (Ishida *et al.*, 1996). In human Intestine 407 cells, hyposmotic stimulation caused activation of ERK1 and ERK2 within 2 min (Tilly *et al.*, 1993). Application of ERK1/2 kinase inhibitor PD98059 prevented activation of hyposmotically stimulated Cl^- current ($I_{Cl,\,vol}$) in rat astrocytes (Crepel *et al.*, 1998), and stimulation of $I_{Cl,\,swell}$ by ET-2 in dog atrial cells (Du and Sorota, 2000). Mechanical stretch (20% for 10 min) strongly stimulated ERKs in AT_{1a} knockout and wild type mice cardiac myocytes (Kudoh *et al.*, 1998).

MAP kinases are activated by GPCRs (Robinson and Dickenson, 2001; Markou *et al.*, 2003; Benoit *et al.*, 2004) and PTKs (Haas *et al.*, 2000; Tahara *et al.*, 2001) in heart cells, making them a possible mediator for the PTK-dependent modulation of I_{Ks} by hyposmotic solution. There are no previous reports on the effects of MAPK kinase inhibitors on native cardiac I_{Ks} or on expressed KCNQ1 channels.

In the present study, a specific ERK1/2 MAPK kinase (MEK) inhibitor PD98059 (Davies *et al.*, 2000) was used in a pretreatment protocol to ensure the maximal inhibition of kinase. The drug had no apparent effect on basal I_{Ks} amplitude. The response of myocytes pretreated with PD98059 to hyposmotic solution was similar to that obtained under control (no drug) conditions, suggesting that MEK was not involved in the responses of I_{Ks} to hyposmotic stimulation. In addition, a short series of experiments was performed using an acute (5–7 min) application of 50 μM PD98059 under hyposmotic conditions, and no significant effect of the drug on hyposmotically-stimulated I_{Ks} amplitude was detected. Taken together, it appears that I_{Ks} is not

modulated by MEK under either basal or hyposmotic conditions. The possible involvement of the two other major subtypes of MAP kinase, p38 and JNK remains to be tested. Using *c-fos* reporter gene constructs, Sadoshima *et al.* (1996) demonstrated that JNK was activated only after 15 min exposure of cultured cardiac myocytes to hyposmotic solution, whereas p38 kinase activity was unaffected.

4.5 Lack of involvement of PI3-K, G-proteins, and the cytoskeleton in the modulation of I_{Ks} by hyposmotic solution

4.5.1 PI3-K and PIP_2

Previous reports on the involvement of PI3-K and PIP_2 in the regulation of cardiac I_{Ks} and expressed KCNQ1 channels contain conflicting findings. Lowering of PIP_2 production by prolonged treatment with wortmannin (50 μM) stimulated I_{Ks} in guinea pig atrial myocytes, whereas loading of cells with PIP_2 inhibited it (Ding *et al.*, 2004). By contrast, application of PIP_2 slowed the rundown of expressed KCNQ1/KCNE1 current (Loussouarn *et al.*, 2003), and rescued loss of function KCNQ1 channel mutants (Park *et al.*, 2005). The results obtained in the present study seem to indicate a tonic PI3-K-dependent modulation of cardiac I_{Ks}, i.e., application of PI3-K inhibitor LY294002 (10 μM) inhibited the basal current amplitude. However, pretreatment with the inhibitor had little effect on the hyposmotic stimulation of I_{Ks}.

4.5.2 G-proteins

The involvement of G-proteins in the stimulation of I_{Ks} by hyposmotic solution was investigated by dialysis of myocytes with a non-hydrolyzable GDP analogue, GDPβS, a compound that prevents exchange of GDP to GTP on α-subunits of G-proteins and "locks" them in the inactive state (Gilman, 1987). The pretreatment of myocytes with GDPβS failed to affect hyposmotic stimulation of I_{Ks}, suggesting that G-proteins are not involved in modulation of I_{Ks} by hyposmotic solutions. The result, however, may be obscured by the fact that GDPβS suppresses both stimulatory (e.g., G_s) and inhibitory (G_i) G-proteins, and modulation of I_{Ks} likely depends on the balance between different types of G-proteins.

4.5.3 Cytoskeleton

The cytoskeleton is considered to be an essential cell structure that is involved in osmotic signaling. Agents that disrupt the cytoskeleton, for example, cytochalasin D, inhibit effects of hyposmotic solution on Ca^{2+}-activated K^+ channels (Grunnet *et al.*, 2002), $I_{Cl,\,swell}$ (Wei *et al.*, 2000), $I_{Ca,\,T}$ (Pascarel *et al.*, 2001), epithelial Na^+ channels (Rehn *et al.*, 1998), and voltage-operated calcium channel current carried by Ba^{2+} (I_{Ba}) (Xu *et al.*, 1997).

In the present study, pretreatment of myocytes with 10 μM cytochalasin D had no effect on the amplitude of I_{Ks} under isosmotic conditions. I_{Ks} in cytochalasin -pretreated myocytes responded to hyposmotic solution in a normal manner. This result is in line with the lack of effect of the inhibitor on inflation-induced stimulation of cardiac I_K (Wang *et al.*, 1996). Similarly, cytochalasin D had little effect on basal KCNQ1/KCNE1 current in COS-7 cells (Loussouarn *et al.*, 2003). On the other hand,

pretreatment of *Xenopus* oocytes with 1 μM cytochalasin D abolished the hyposmotic stimulation of of KCNQ1 current (Grunnet *et al.*, 2003).

Recently, activation of $I_{\text{Cl, swell}}$ in rabbit ventricular myocytes has been linked to integrin/focal adhesion kinase (FAK)-related signaling (Browe and Baumgarten, 2003). Integrins are membrane-bound heterodimers that are capable of transmission of a variety of signals from the extracellular matrix (ECM) to the cytoskeleton and are thought to be a type of eukaryotic mechano- and osmo-sensor (Ishida *et al.*, 1996; Sadoshima *et al.*, 1996; Lehoux and Tedgui, 1998). It is known that PTK plays an important role in integrin–cytoskeleton signaling. Upon stimulation, integrins bind FAK which, in turn, binds and activates members of the Src family of tyrosine kinase that transmit signals to several downstream pathways, including PKC, PI3-K, and small GTPases (Parsons, 2003). Indeed, stimulation of β1-integrin by mechanical stress caused activation of $I_{\text{Cl, swell}}$ via an Src-dependent pathway in rabbit ventricular myocytes (Browe and Baumgarten, 2003).

Integrin signaling requires a specific cytoskeleton organization, and cytoskeleton disruption by cytochalasin D suppresses this mechanism (Wei *et al.*, 2000). In the present study, I_{Ks} was stimulated by hyposmotic solution in myocytes pretreated with 10 μM cytochalasin D. Therefore, it seems unlikely that integrin signaling is a key factor in this stimulation.

4.6 Regulation of I_{Ks} by tyrosine phosphorylation

4.6.1 Modulation of basal I_{Ks} by PTK inhibitors

The amplitude of basal I_{Ks} was markedly reduced by the application of PTK inhibitors. For example, genistein (50 μM) reduced the amplitude by an average 44%, a value that is similar to the 46% reduction observed by Zhou *et al.* (1997) in an earlier study on canine ventricular myocytes. The most important question that arises from the inhibition of I_{Ks} by genistein is whether it was caused by an inhibition of PTK activity or not. In that regard, there is no shortage of earlier studies suggesting that genistein can have a direct blocking action on ion channels. The usual criterion used for evaluation of direct blocking effect is whether PTK-inactive analogue daidzein has a similar blocking action. For example, both genistein and daidzein inhibited I_{Ks} in guinea pig ventricular myocytes (Washizuka *et al.*, 1997, 1998; Matsubayashi *et al.*, 1999), and voltage-sensitive Na^+ current in cultured rat brain neurons (Paillart *et al.*, 1997).

In the present study, the results obtained with genistein and its inactive analogues are consistent with a PTK-mediated mechanism of genistein action on I_{Ks}. Genistein inhibited I_{Ks} with an EC_{50} of 64 $\pm$ 3.8 μM, daidzein had a much weaker inhibitory effect, and PTK-inactive genistin had no effect at concentrations up to 200 μM.

The two other broadspectrum PTK inhibitors used in the present study were A23 and A25. To my knowledge, there are no previous reports on the effects of A23 and A25 on cardiac I_{Ks}. Both of these compounds rapidly and reversibly reduced the amplitude of I_{Ks} (EC_{50} values of 4 and 12 μM for A23 and A25, respectively). By

contrast, neither PTK-inactive A1 nor PTK-inactive A63 (50–100 μM) had significant inhibitory effects on the current. These results suggest that PTK has a strong regulatory action on basal I_{Ks} in guinea pig ventricular myocytes.

4.6.2 PTK inhibitors attenuate the stimulation of I_{Ks} by hyposmotic solution

In addition to their inhibitory actions on basal I_{Ks}, broadspectrum PTK inhibitors genistein, A23, and A25 attenuated the stimulation of I_{Ks} by hyposmotic solution. Suppression of I_{Ks} stimulation by genistein (50 μM) has been previously reported in a study on canine ventricular myocytes (Zhou *et al.*, 1997). In that study, PTK-inactive daidzein had a weak inhibitory effect on basal current amplitude, and failed to prevent hyposmotic stimulation of the current. Quite a different result was obtained in a study on KCNQ1 channels expressed in COS-7 cells (Kubota *et al.*, 2002): stimulation of KCNQ1 current by hyposmotic solution was unaffected by pretreatment with 50 μM genistein. Moreover, substitution of ATP in the pipette solution with a nonhydrolyzable analogue, AMP-PNP, did not affect hyposmotic stimulation of KCNQ1 current, suggesting that protein phosphorylation was not involved in the response (Kubota *et al.*, 2002). The reasons for the discrepancy between the results obtained by Kubota *et al.* (2002) and those obtained with genistein here could include differences in the sensitivity of native versus expressed channels to modulation by PTK, as well as differences in the activity of signaling molecules in myocytes and COS-7 cells. The effects of ATP analogues on stimulation of I_{Ks} in cardiac myocytes and expressed KCNQ1 channels by 0.75T solution remain to be tested.

The inhibition of PTK by genistein, A23, and A25 may affect the level of tyrosine

phosphorylation of channel proteins and/or signaling molecules upstream from the channels. For example, tyrosine phosphorylation can cause modulation of the activity of serine/threonine protein phosphatases (Chen *et al.*, 1992), MAP kinase (Hagemann and Blank, 2001; Cussac *et al.*, 2002), PTK (Bokemeyer *et al.*, 2000; Daub *et al.*, 1996), and PTP (Vogel *et al.*, 1993; Liu and Chernoff, 1997). Therefore, it is possible that stimulation of I_{Ks} by hyposmotic solution is not mediated by direct tyrosine phosphorylation of channels, but by PTK-dependent modulation of the secondary signaling pathway.

Another possible mechanism of modulation of Ks channel activity by tyrosine phosphorylation is via modulation of channel trafficking. For example, it has been reported that suppression of Kv1.2 channel activity by stimulation of PTK is due to a stimulation of the endocytosis of channel α subunits (Nesti *et al.*, 2004). Similarly, inhibition of PTP has been linked to an increase in the endocytosis of apical K^+ channels in rat cortical collecting duct cells (Wei *et al.*, 2000) and renal outer medullary potassium channel 1 (ROMK1) (Sterling *et al.*, 2002). On the other hand, an increase in PTK activity by insulin lead to an increase in the exocytosis and consequent strong and rapid (within 10 min) stimulation of current through N-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes (Skeberdis *et al.*, 2001). In case of KCNQ1 channels, tyrosine residue Y111 is important for membrane localization of channels expressed in MDCK cells: when this residue was mutated into alanine, the KCNQ1 current was absent due to the cytoplasmic distribution of mutated channels (Jespersen *et al.*, 2004). It is, therefore, possible that stimulation of I_{Ks} by hyposmotic solution was due to stimulation of a PTK-dependent *exocytosis* of Ks channel subunits, and that inhibition of I_{Ks} stimulation by PTK inhibitors was due

to inhibition of the exocytosis. However, the time course of hyposmotic stimulation of I_{Ks} was quite rapid (a noticeable change in current amplitude within 1–3 min). It is known that hyposmotic stress can activate tyrosine kinase within 5 s in rat cardiac myocytes (Sadoshima *et al.*, 1996). Channel trafficking, on the other hand, usually occurs with a lag of 5–10 min (Zhou *et al.*, 2000; Feranchak *et al.*, 2003). However, there are reports of faster increases in C_m and membrane conductance (e.g., a change within 1 min from the onset of hyposmotic stress (Okada *et al.*, 1992)).

It is not clear whether a particular PTK can play the role of the osmotic sensor in cardiac myocytes, or whether its activity is modulated by other osmosensitive mechanisms (for example, macromolecular crowding). Although there are no published reports on the effects of macromolecular crowding on PTK activity, it is a possibility that is not easily discounted.

4.6.3 Reversal of the effects of PTK inhibitors by vanadate compounds

In order to confirm that the effects of PTK inhibitors on I_{Ks} were related to the suppression of PTK activity, a series of experiments were performed with Van and DMHV. Van (Swarup *et al.*, 1982; Gordon, 1991) and DMHV (Cuncic *et al.*, 1999; Hsu *et al.*, 2003) are PTP inhibitors that have previously been used to modulate tyrosine phosphorylation on a variety of K^+ channels (Holmes *et al.*, 1996b; Gamper *et al.*, 2000; Imbrici *et al.*, 2000; Zhang and Wang, 2000; Gao *et al.*, 2004). Under isosmotic conditions, both drugs rapidly restored the genistein-, A23-, and A25-inhibited I_{Ks} amplitude to ≈90% of control value. A similar degree of restoration of I_{Ks} amplitude was also obtained under hyposmotic conditions in myocytes that were pretreated with

A23 and A25. In previous studies on non-I_{Ks} currents, Van potentiated swelling-activated chloride current inhibited by genistein and tyrphostins A25 and B46 in bovine endothelial cells (Voets *et al.*, 1998). By contrast, volume-sensitive Cl^- current in bovine chromaffin cells was inhibited by pervanadate and unaffected by tyrphostin B46 and genistein (Doroshenko, 1998).

It is possible that the stimulatory effects of Van and DMHV on I_{Ks} in myocytes pretreated with PTK inhibitors were mediated via a non-PTP action. For example, there are studies that indicate that Van can stimulate adenylate cyclase and thereby stimulate the cAMP–PKA pathway (e.g., Tsiani and Fantus (1997)). However, neither Van nor DMHV stimulated basal I_{Ks}, suggesting that myocyte adenylate cyclase was not stimulated by these compounds.

Van and DMHV did not affect the amplitude of basal I_{Ks}, but had strong stimulatory effects on the current in myocytes pretreated with PTK inhibitors. On the other hand, it has been reported that Van increased I_{Ba} under both isosmotic and hyposmotic conditions in canine basilar arterial myocytes (Kimura *et al.*, 2000), and increased the efflux of ions ($^{125}I^-$ and $^{86}Rb^+$) in human Intestine 407 cells under mild hypotonic conditions (Tilly *et al.*, 1993). A possible interpretation of the lack of effect of PTP inhibitor on the current amplitude under basal conditions is that the basal activity of PTP in myocytes is very low, such that application of PTP inhibitor does not significantly increase tyrosine phosphorylation on Ks channel protein (or pertinent channel-regulatory protein). A similar lack of effect of vanadate compounds under basal conditions (and reversal of the effects of PTK inhibitors by PTP inhibitors) has previously been reported in studies on the involvement of tyrosine phosphorylation in the regulation of $I_{Ca,\,L}$ in guinea pig ventricular myocytes (Ogura *et al.*, 1999) and

Kv1.4 channels expressed in CHO cells (Zhang and Wang, 2000).

If basal PTP activity is low, how can application of a PTP inhibitor increase tyrosine phosphorylation in myocytes pretreated with PTK inhibitors? One possibility is that application of PTK inhibitor not only decreases PTK activity, but also stimulates PTP activity via a decrease of inhibitory tyrosine phosphorylation on PTP, which, in turn, is sensitive to PTP inhibitor. It has been speculated that such a mechanism may exist in cultured rat osteoclasts, where genistein markedly increased PTP activity (Gao and Yamaguchi, 2000). However, it is also important to note that the converse mechanism has also been reported, i.e., Vogel *et al.* (1993) and Liu and Chernoff (1997) found that PTP1B was tyrosine phosphorylated by EGFR kinase, leading to increased PTP activity.

Returning to the possibility that inhibition of PTK stimulated PTP activity via a facilitation of dephosphorylation of the PTP, how might the actual dephosphorylation occur? It could be carried out either by trans- dephosphorylation by another PTP ("PTP_x"), or by an autodephosphorylation process, such as autodephosphorylation of phosphotyrosyl residues on the PTP SHP-2 (Kontaridis *et al.*, 2004) and PTP1D (Stein-Gerlach *et al.*, 1995).

Pretreatment of myocytes with very high concentrations of A23 and A25 abolished the stimulatory action of PTP inhibitors. This is the result expected from modulation of Ks channels by tyrphostins because high concentrations of A23 and A25 are likely to have lowered PTK activity to a level insufficient for promotion of a sizeable level of tyrosine phosphorylation in the presence of PTP inhibitor.

4.6.4 Attenuation of hyposmotic stimulation of KCNQ1/KCNE1 current by A25

In the present study, tyrphostin A25 (20 μM) attenuated the hyposmotic stimulation of KCNQ1/KCNE1 current in BHK cells. Thus, it appears that tyrosine phosphorylation of either channel proteins or upstream signaling molecules is a key factor in the hyposmotic stimulation of expressed KCNQ1/KCNE1 channels in BHK cells. However, this result is at odds with the finding that 50 μM genistein failed to prevent the responses of expressed KCNQ1 and KCNQ1/KCNE1 to hyposmotic solutions in COS-7 cells (Kubota *et al.*, 2002). The reason for the discrepancy may be related to the difference in the expression system used in the two studies.

4.6.5 Lack of involvement of EGFR kinase

The involvement of EGFR kinase in cell signaling processes induced by changes in osmolarity is well established. For example, EGFR inhibitor AG1478 suppressed hyperosmotically-induced phosphorylation of cytosolic phospholipase A_2 in HaCaT cells (Rodriguez *et al.*, 2002), EGFR inhibitor tyrphostin B46 (but not PDGFR-specific inhibitor tyrphostin AG1296) inhibited volume-sensitive Cl^- currents in murine mammary cells (Abdullaev *et al.*, 2003), and tyrphostin AG1478 inhibited the activation of swelling-activated ion and taurine transport in human cervical cancer cells (Shen *et al.*, 2001). In addition, tyrphostin AG1478 and EGFR/JAK2 inhibitor B42, but not PDGFR inhibitor AG1295, suppressed stretch-induced ERK activation in mice cardiac myocytes (Kudoh *et al.*, 1998).

In the present study, AG1478 had little effect on I_{Ks} amplitude under basal conditions, and did not prevent stimulation of I_{Ks} by hyposmotic solution. The latter

result suggests that EGFR kinase is not involved in hyposmotic stimulation of I_{Ks}.

4.6.6 Involvement of Src kinase in the regulation of I_{Ks}

There are no previous reports on the involvement of Src-type PTK in the regulation of cardiac I_{Ks}. In the present study, Src inhibitor PP2 (Hanke *et al.*, 1996) strongly and reversibly inhibited the amplitude of basal I_{Ks}, whereas inactive analogue PP3 was without effect. On the other hand, (Gamper *et al.*, 2003) reported that overexpression of Src kinase failed to affect the activity and tyrosine phosphorylation level of KCNQ1 channels expressed in CHO cells.

In addition to its effects on basal I_{Ks}, PP2 attenuated the response of the current to hyposmotic solution. Previous studies indicate that stimulation of Src kinase activity by application of Src-activating peptide enhances $I_{Cl,\,vol}$ in rabbit nonpigmented ciliary epithelial cells (Shi *et al.*, 2002) and in human lymphocytes (Lepple-Wienhues *et al.*, 2000), and that direct mechanical stimulation of rabbit ventricular myocytes stimulated $I_{Cl,\,vol}$ via an Src-dependent pathway (Browe and Baumgarten, 2003). On the other hand, inhibition of Src stimulated $I_{Cl,\,vol}$ in human atrial myocytes (Du *et al.*, 2004).

The results obtained in the present study suggests that cardiac I_{Ks} is under strong regulation by tyrosine phosphorylation, and that cytoplasmic PTK (Src family) is involved in the regulation of the current.

4.6.7 Possible mechanisms involved in PTK/PTP-dependent modulation of cardiac I_{Ks} under isosmotic and hyposmotic conditions

A schematic presentation of possible tyrosine phosphorylation/dephosphorylation dependent mechanisms involved in the modulation of I_{Ks} under isosmotic and hyposmotic conditions is provided in Figure 33.

The results obtained in the present study suggest that Ks channels in guinea pig ventricular myocytes are constitutively tyrosine-phosphorylated under basal conditions and that tyrosine kinase activity is required for the optimal function of these channels. Although PTP inhibitors were ineffective under basal conditions, it appears that their action was enhanced when PTK activity was inhibited. Such a result can be explained by (i) the presence of an inhibitory influence of PTK on PTP activity, or (ii) much higher activity of PTK compared to PTP under basal conditions, and very potent inhibitory action of PTK inhbitors that lowers PTK activity below that of PTP. In future work, obtaining dose–response relationships for PTP inhibitor action on I_{Ks} amplitude in myocytes pretreated with different concentrations of A25 or A23 can help to distinguish between the two possibilities. If the EC_{50} of Van effect on I_{Ks} increases with increasing concentration of A25, the inhibitory influence of PTK on PTP activity is likely.

It appears that stimulation of I_{Ks} by hyposmotic solution depends on Src-type PTK activity. PTK (Src) under hyposmotic conditions may (i) directly tyrosine phosphorylate Ks channels, thereby stimulating I_{Ks}, (ii) modulate a cellular signaling pathway which results in I_{Ks} stimulation, or (iii) play a role in Ks channel exocytosis. The first possibility, i.e., a direct phosphorylation of Ks channels by (postulated)

hyposmotic-activated Src, is at odds with the lack of detected tyrosine phosphorylation on expressed KCNQ1 upon overexpression of Src (Gamper *et al.*, 2003). In addition, if Ks channels are strongly tyrosine phosphorylated under basal conditions (in part because of the apparent low activity of PTP), how would an increase in PTK activity induced by hyposmotic swelling cause a near-doubling of the amplitude of I_{Ks}? One possibility is that stimulation of PTK under hyposmotic conditions may lead to phosphorylation of a secondary, low affinity, tyrosine site on Ks channel protein which increases channel open probability. However, the rapid decrease of I_{Ks} amplitude that occurs upon removal of hyposmotic stimulation in the presence of Van, fails to support the proposed hypothesis. A definitive answer could be obtained by comparison of Western blot analysis of tyrosine phosphorylation levels on KCNQ1 protein under isosmotic and hyposmotic conditions.

Regarding the possibility of an indirect stimulation of I_{Ks} by a PTK-dependent mechanism, one possible mediator with a known effect on I_{Ks} is PIP_2 (whose production in HEK-293 cells is increased by augmentation of tyrosine phosphorylation (Rumenapp *et al.*, 1998)). In future experiments, the intracellular application of PIP_2 or PIP_2 scavenging agent poly-L-lysine (Loussouarn *et al.*, 2003) under both isosmotic and hyposmotic conditions may help to elucidate a possible role in hyposmotic stimulation of I_{Ks}.

As discussed earlier, hyposmotic swelling may dramatically increase the rate of Ks channel exocytosis to the plasma membrane. If this is the case, two scenarios are possible. First, the rate of exocytosis may increase in a PTK-independent manner. However, it seems likely that the trafficked channels then need to be tyrosine phosphorylated in order to become operational. Therefore, the degree of hyposmotic

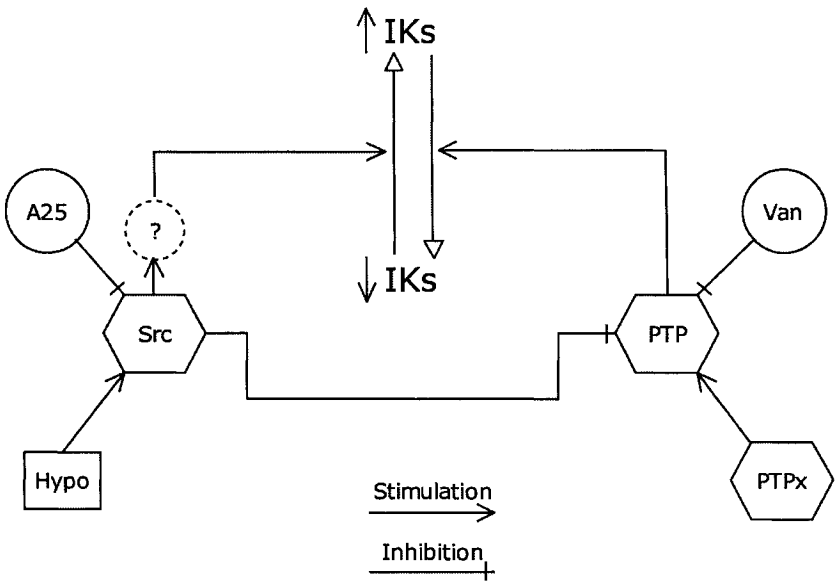

Figure 33: Schematic of possible PTK/PTP-dependent mechanisms involved in the modulation of I_{Ks} under isosmotic and hyposmotic conditions.

stimulation of I_{Ks} amplitude in myocytes pretreated with PTK inhibitor should be similar to that in control myocytes (e.g., 73% increase with 0.75T), because the number of inserted channels depends only on the degree of hyposmotic stimulation, not on the level of myocyte PTK activity. In fact, the relative increase of I_{Ks} in pretreated myocytes was substantially smaller (e.g., ≈20% for 20 μM A25). This finding points to a PTK-dependent exocytosis in which the number of newly-inserted channels increases when PTK activity is high, and decreases when it is low. The degree of I_{Ks} stimulation by hyposmotic solution in myocytes pretreated with PTK inhibitors will then be lower due to the lower number of channels inserted in the plasma membrane. If trafficking is the mechanism of hyposmotic stimulation of I_{Ks}, then both exocytosis of channels during hyposmotic myocyte swelling and endocytosis upon return to isosmotic conditions, should be quite rapid and correspond to the observed rapid change in the amplitude of I_{Ks}. If trafficking is a PTK-dependent process, why does pretreatment of myocytes with Van not sustain the stimulated I_{Ks} upon removal of hyposmotic solution? A simple explanation may be that exocytosis, but not endocytosis, of channel proteins is controlled by PTK/PTP activity. One way of testing this possibility would be by imaging expressed KCNQ1/KCNE1 channel distribution in control and PTK inhibitor-pretreated cells using confocal immunofluorescence microscopy (Vessey *et al.*, 2004; Leung *et al.*, 2005), under both basal and hyposmotic conditions. It is also possible to test for the degree of stimulation of I_{Ks} under hyposmotic conditions in cells pretreated with exocytosis inhibitor brefeldin A (Nebenfuhr *et al.*, 2002) and endocytosis inhibitor concanavalin A (Wei *et al.*, 2000).

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