University of Alberta

Effects of Dietary Polyphenols on Cardiac Ion Channels

by

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Abstract

Evidence suggesting polyphenols may modulate ion channels independently of antioxidant activity prompted an investigation into ion channel inhibition by dietary polyphenols and potential cardioprotection via this mechanism.

Using the patch-clamp technique, the effects of grape extract and three grape polyphenols, resveratrol, quercetin and catechin, were examined in recombinantly expressed $Na_V1.5$ voltage-gated sodium channels, and were found to block peak current. Quercetin and resveratrol also blocked late current, which is clinically significant in Long QT syndrome and ischemia/reperfusion injury. The effects of grape extract, resveratrol and the green tea polyphenol epigallocatechin gallate were investigated in recombinant $K_V1.5$ potassium channels, which they were found to block, clinically relevant to atrial fibrillation.

To further characterize late I_{Na} block as a mechanism of cardioprotection by polyphenols, experiments were performed in a rat cardiomyocyte model of increased late I_{Na}. Inhibition of late I_{Na} by resveratrol led to reductions in cardiomyocyte calcium overload and contractile dysfunction.

Dietary polyphenols thus have sodium- and potassium-channel blocking effects that may offer cardioprotection in a variety of pathophysiological settings.

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Dedication

This thesis is dedicated to all those who have provided me with support and inspiration, especially:

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List of abbreviations and symbols

([ADP]) ADP	(concentration of) adenosine diphosphate
([ATP]) ATP	(concentration of) adenosine triphosphate
([Ca^{2+}]) Ca^{2+}	(concentration of) calcium ion
([H^+]) H^+	(concentration of) hydrogen ion
([K^+]) K+	(concentration of) potassium ion
([Na^+]) Na^+	(concentration of) sodium ion
([P_i]) P_i	(concentration of) inorganic phosphate
$[ATP]_i$	intracellular adenosine triphosphate concentration
$[ATP]_o$	extracellular adenosine triphosphate concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_{mito}$	intramitochondrial calcium concentration
$[Ca^{2+}]_o$	extracellular calcium concentration
$[K^+]_i$	intracellular potassium concentration
$[K^+]_o$	extracellular potassium concentration
$[Na^+]_i$	intracellular sodium concentration
$[Na^+]_o$	extracellular sodium concentration
A	alanine
***Acc*65I**	restriction endonuclease (G/GTACC cutsite)
ACh	acetylcholine
AF	atrial fibrillation
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AP(D)	action potential (duration)
ATXII	*Anemonia sulcata* (sea anemone) toxin type II
Aβ	beta-amyloid
bp	base pair
BSA	bovine serum albumin
***Bsr*GI**	restriction endonuclease (T/GTACA cutsite)
$CaCl_2$	calcium chloride

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CHD	coronary heart disease
CHF	congestive heart failure
CO_2	carbon dioxide
CsCl	cesium chloride
CsOH	cesium hydroxide
C-terminus	carboxyl terminal end
D	aspartate
DI-DIV	domain one to domain four
E	glutamate
EGCG	epigallocatechin gallate
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
E_K	potassium equilibrium potential
E_m	membrane potential
eNOS	endothelial nitric oxide synthase
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
F	phenylalanine
G	glycine
GFP	green fluorescent protein
gIRK	strong inward rectifier potassium channel
GIT	gastro-intestinal tract
$g_{max(50)}$	maximal conductance (potential at half-maximal conductance)
HEK-293	human embryonic kidney cell line 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
hERG	human ether-a-go-go related gene
hH1a	human heart voltage-gated sodium channel clone
Hz	hertz (cycles per second)
I	isoleucine
i.p.	intraperitoneal
I/R	ischemia/reperfusion
IC_{50}	inhibitory concentration required for half-maximal inhibition

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$I_{Ca,L}$	L-type calcium current
I_{Cl}	chloride current
$I_{Cl(Ca)}$	calcium-activated chloride current
IHD	ischemic heart disease
$I_{K,slow1}$	rapidly activating, slowly inactivating potassium current
$I_{K,slow2}$	slowly inactivating outward potassium current
I_{K1}	strong inward rectifier potassium current
I_{KAA}	arachidonic acid-activated potassium current
I_{KACh}	acetylcholine-activated potassium current
I_{KATP}	adenosine triphosphate-sensitive potassium current
I_{KNa}	sodium-activated potassium current
I_{Kp}	plateau potassium current
I_{Kr}	rapid delayed rectifier potassium current
I_{Ks}	slow delayed rectifier potassium current
I_{Kur}	ultra-rapid delayed rectifier potassium current
I_{Na}	sodium current
$I_{Na/Ca}$	sodium-calcium exchanger current
iNOS	inducible nitric oxide synthase
I_{NSC}	non-selective cation current
I_{ss}	steady-state potassium current
I_{to}	transient outward potassium current
$I_{to,f}$	fast transient outward potassium current
$I_{to,s}$	slow transient outward potassium current
K	lysine
K_{ATP}	adenosine triphosphate-sensitive potassium channel
KCl	potassium chloride
KCNE1	rapid delayed rectifier voltage-gated potassium channel β-subunit gene
KCNE2	slow delayed rectifier voltage-gated potassium channel β-subunit gene
KCNH2	rapid delayed rectifier voltage-gated potassium channel α-subunit gene
KCNQ1	slow delayed rectifier voltage-gated potassium channel α-subunit gene
KOH	potassium hydroxide

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$K_V1.5$	ultra-rapid delayed rectifier voltage-gated potassium channel α-subunit protein
$K_V11.1$	rapid delayed rectifier voltage-gated potassium channel α-subunit protein
$K_V7.1$	slow delayed rectifier voltage-gated potassium channel α-subunit protein
KvLQT1	slow delayed rectifier voltage-gated potassium channel α-subunit
L	myocyte cell length
LCAC	long-chain acylcarnitines
LDL	low-density lipoprotein
Lo	starting myocyte cell length
LQTS (LQT1-LQT6)	long QT syndrome (type one to type six)
M cells	cardiomyocytes of the midmyocardial layer
M	methionine
MCS	multiple cloning site
MgATP	magnesium adenosine triphosphate
$MgCl_2$	magnesium chloride
minK	slow delayed rectifier voltage-gated potassium channel β-subunit
MiRP1	rapid delayed rectifier voltage-gated potassium channel β-subunit
mRNA	messenger ribonucleic acid
mV	millivolts
M	megaohms
N	asparagine
n	sample size
N1325S	mutation of asparagine to serine at residue 1325
nA	nanoamps
Na^+/HCO_3^-	sodium bicarbonate cotransporter
Na^+/K^+-ATPase	adenosine triphosphate-driven sodium-potassium exchange pump
NAc	N-acetylcysteine
NaCl	sodium chloride
NaOH	sodium hydroxide
$Na_V1.5$	voltage-gated sodium channel protein
NCX	sodium-calcium exchanger
NFκB	nucleotide factor kappa b

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NHE	sodium-hydrogen exchanger
***Nhe*1**	restriction endonuclease (G/CTAGC cutsite)
N-terminus	amino terminal end
P	probability
P	proline
pA	picoamps
pcDNA	plasmid (vector) circular deoxyribonucleic acid
PCR	polymerase chain reaction
pfu	plaque-forming units
pGL	green fluorescent protein plasmid
pH_i	intracellular hydrogen potential
pH_o	extracellular hydrogen potential
PKA	protein kinase isoform a
PKC	protein kinase isoform c
PLC	phospholipase isoform c
P-loop	pore-lining loop
PTK	protein tyrosine kinase
Q	glutamine
R	arginine
R1193Q	mutation of arginine to glutamine at residue 1193
R1623Q	mutation of arginine to glutamine at residue 1623
R1644H	mutation of arginine to histidine at residue 1644
S	serine
S1-S6	segments one to six
SCN5A	voltage-gated sodium channel alpha-subunit gene
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
SV-40	simian virus 40
T	threonine
TEA-Cl	tetraethylammonium chloride
tsA201	simian virus 40-transformed human embryonic kidney cell line 293

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v v^{-1}	volume per volume
V	valine
VGPC	voltage-gated potassium channel
VGCC	voltage-gated calcium channel
VGSC	voltage-gated sodium channel
w v^{-1}	weight per volume
w w^{-1}	weight per weight
WT	wild type
Y	tyrosine
ΔKPQ	deletion at lysine 1505, proline 1506 and glutamine 1507
Δ*L*	change in myocyte cell length

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INTRODUCTION

Cardiac ion channels

Communication within and between cells is essential for life at the tissue, organ and organism levels. There are many ways this is achieved, including through endocrine and neuroendocrine signaling; enzyme- and receptor-based second messenger cascades; and electrical communication via ion currents through channels, pumps and exchangers. In the heart, effective coordination of action potential conduction between myocytes, and of calcium signal regulation within myocytes, conveys the pace and the magnitude of cardiac contractions.

Concentration gradients of various ions in the cardiomyocyte are maintained by ion transporters and generate ion currents that control the resting membrane potential and the excitability of the cell. The resting concentration of potassium ions is maintained at ~ 150 mM intracellularly, compared to an extracellular value of ~ 4 mM; therefore, according to the Nernst equation, the potassium equilibrium potential (E_K) is – 96 mV. Sodium concentrations inside and outside the cell are ~ 20 mM and 145 mM, respectively, generating an equilibrium potential (E_{Na}) of + 50 mV; A gradient of 2.5 mM extracellular to 0.001 mM intracellular calcium gives an equilibrium potential (E_{Ca}) of + 134 mV. Due to the greater permeability of potassium ions through gIRK channels and other strong inward rectifiers, compared with sodium and calcium ions through their respective channels and ion pumps, and charge separation between ionic species and membrane-impermeable intracellular anions, the resting membrane potential (E_m) of the cell will be negative and close to E_K, at around – 85 mV. The electrochemical driving

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forces acting on these ions dictate that increases in permeability will generate outward potassium currents and inward sodium and calcium currents (Klabunde, 2004).

Many ionic currents in cardiac conductive fibres and in the myocardium control the shape of the action potential (Figure 1). Net inward sodium current (peak I_{Na}) via the voltage-gated sodium channel (VGSC) generates, and is normally limited to, the fast depolarization phase (Phase 0) of the action potential. Using the example of a human ventricular action potential, after this rapid upstroke and a brief downward deflection caused by transient outward potassium current (I_{to}) (Phase 1), there is a plateau (Phase 2) maintained principally by calcium channel currents and sodium-calcium exchanger (NCX) currents ($I_{Na/Ca}$) opposing outward delayed rectifier potassium currents such as I_{Ks} and I_{Kr}. The repolarization phase (Phase 3) is produced when the latter currents become unopposed. Strong inward rectifier currents (I_{K1}) are then responsible for the final repolarization of the membrane potential to approximately – 85 mV (Phase 4) in readiness for the arrival of another stimulus (Tamargo *et al.*, 2004).

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Figure 1. The cardiac action potential

a. The typical ventricular action potential is divided into five phases. The upstroke, phase 0, is controlled by inward fast sodium current (peak I_{Na}); the transient repolarization of phase 1 is due to activation of I_{to} potassium channels; phase 2 consists of a plateau where both inward I_{Ca} and $I_{Na/Ca}$ and outward I_{Ks}, I_{Kr} and I_{Kur} currents are active; in phase 3 the delayed rectifier potassium currents I_{Ks}, I_{Kr} and I_{Kur} cause a repolarization of the action potential; and phase 4 represents the resting membrane potential between action potentials, maintained close to the potassium equilibrium potential by I_{K1} currents through strong inward rectifier potassium channels. **b.** The QRS complex of the electrocardiogram corresponds to the depolarization of the ventricular action potential while the T wave corresponds to its repolarization. The QT interval may be increased in cases of prolonged action potential duration.

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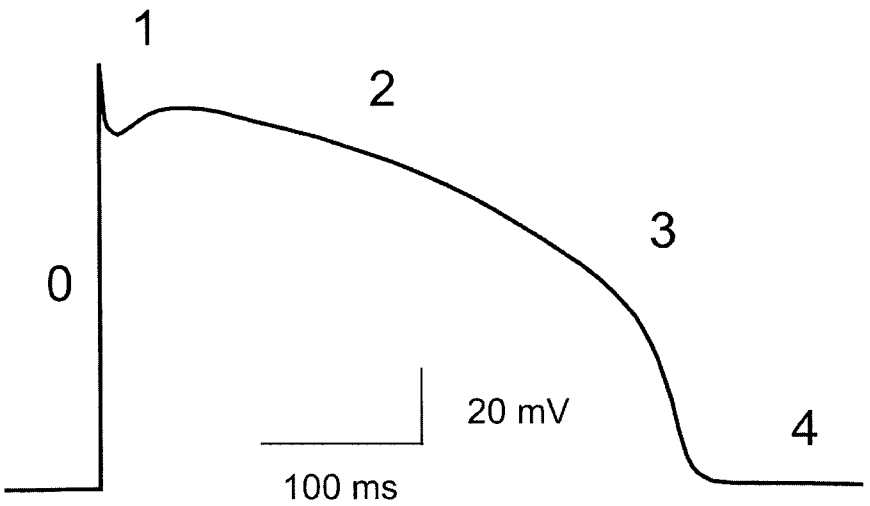
a.



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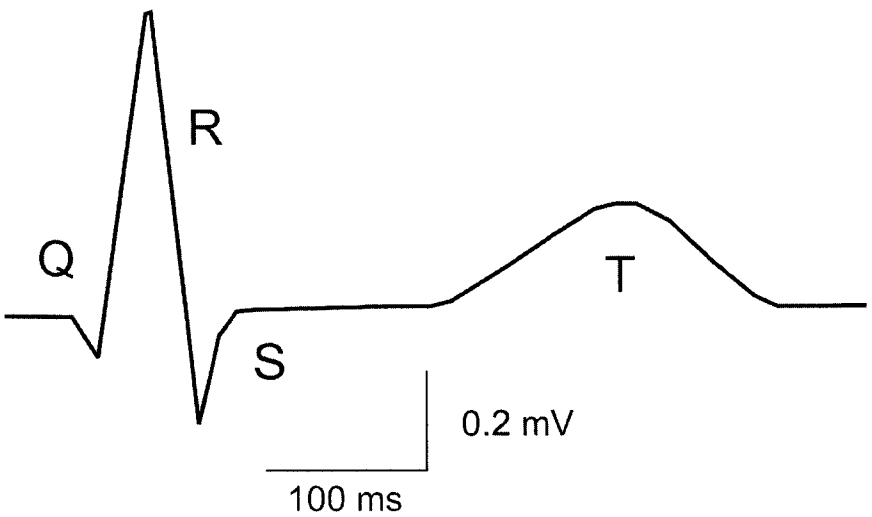

Figure 1.

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Voltage-gated sodium channels

The voltage-gated sodium channel (VGSC) is a membrane-bound protein composed of an α subunit that makes up the ion-conducting pore, and one of several modulatory β subunits. The α subunit consists of four domains (DI – DIV) of six α-helical membrane-spanning segments (S1 – S6) (Figure 2a). The fourth segment (S4) of each domain makes up part of a voltage sensor containing positively charged residues that respond to potential differences across the lipid bilayer of the plasma membrane. The S5 and S6 segments and the loop between them (P-loop) line the pore formed by the symmetrical arrangement of the four domains. Each P-loop contributes one amino acid (D, E, K and A from domains DI, DII, DIII and DIV respectively) to a filter that selects for the passage of sodium cations only. The alkylammonium of the lysine residue in DIII is thought to be the principal regulator of this selectivity filter via modulation of cation-cation interactions through the pore (Favre *et al.*, 1996).

The activation of the VGSC first involves movement of the positively charged S4 voltage sensor segments outward in response to cell depolarization. This leads to a conformational change in the S5 and S6 segments and P-loop that allows sodium ions to pass through the selectivity filter. This process of activation takes less than 1 ms, and within 10 ms, while the cell is still depolarized, VGSCs may enter a state of fast inactivation when a group of three amino acid residues in the linker between domains DIII and DIV (I, F and M) binds to a site on the inside face of the channel, occluding the pore (Figure 2b). Slow inactivation occurs via conformational changes in the P-loops. VGSCs return to the closed state and then recover from inactivation when the IFM peptide dissociates from the channel (Figure 2b) (Catterall, 2000; Grant, 2001). Contrary

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to early findings in the field (Hodgkin & Huxley, 1952), it is now widely held that activation and inactivation are coupled; however, activation and deactivation are much more strongly voltage dependent than inactivation, which is largely time dependent (Aldrich & Stevens, 1987).

The $Na_V1.5$ protein encoded by the SCN5A gene forms the α subunit of the cardiac VGSC. This α subunit interacts with a β subunit to form a fully functional VGSC. The β subunit may modulate expression of VGSCs via cell adhesion activity (McEwen *et al.*, 2004) and their gating via modification of surface charges (Ferrara & Moran, 2006) to produce leftward shifts in the voltage-dependence of activation and inactivation (Isom *et al.*, 1995). As mentioned previously, VGSC activation is responsible for the upstroke of the action potential and inactivation usually occurs within 10 ms. Nevertheless, inactivation or the coupling of activation to inactivation may be impaired in a number of ways, including by free radicals, lipid mediators and enzyme activities resulting from I/R injury (Undrovinas *et al.*, 1992; Baetz *et al.*, 2003; Barrington *et al.*, 1997; Watson & Gold, 1997; Schreibmayer, 1999; Light *et al.*, 2003) or by heritable mutations in SCN5A, as is the case in Long QT syndrome type 3 (LQT3) (Dumaine *et al.*, 1996; Bennett *et al.*, 1995). Alteration of normally rapid I_{Na} inactivation in either of these situations, which will be discussed in greater detail below, may lead to abnormal sustained I_{Na} (late I_{Na}) during a prolonged plateau phase of the action potential, entailing consequences such as arrhythmogenesis or calcium overload via increased reverse-mode sodium-calcium exchange.

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Figure 2. The voltage-gated sodium channel

a. Representation of the four six-transmembrane-segment domains that form the VGSC. The S4 domain contains positively charged residues; the P-loop between S5 and S6 lines the pore; and the inactivation loop between DIII and DIV contains the IFM tripeptide that allows pore occlusion **b.** VGSCs open upon activation and inactivate via a "ball-and-chain" mechanism, involving the inactivation loop occluding the pore. Return to the resting state involves closing of the channel (deactivation) and dissociation of the IFM loop (recovery from inactivation).

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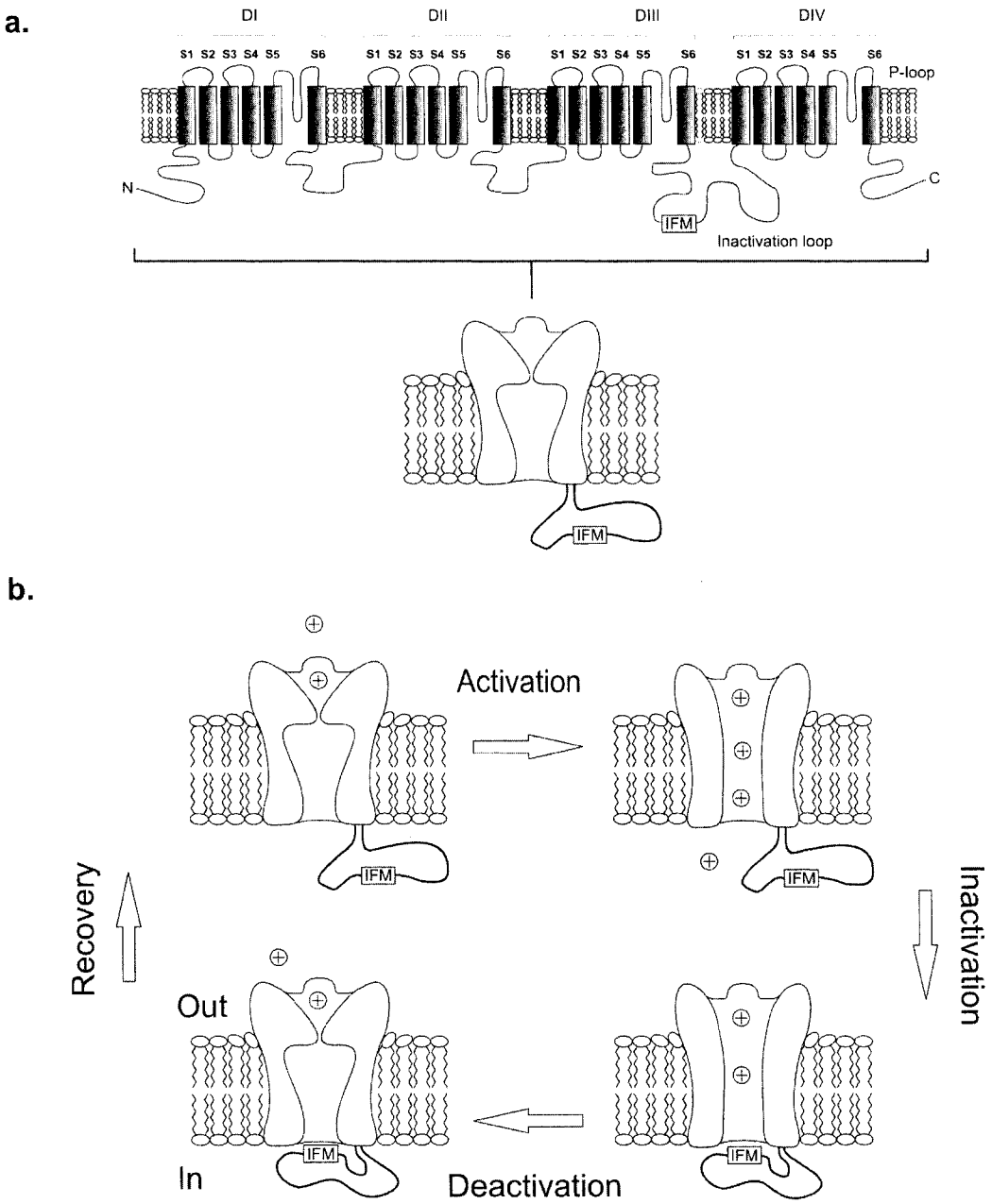

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Voltage-gated potassium channels

Potassium channels are the most ancient and ubiquitous class of ion channel. There are a multitude of types, as distinguished by their primary means of regulation (voltage, $[Ca^{2+}]$, ATP, ACh, among numerous modulators) as well as by their genetic sequence. Voltage-gated potassium channels are formed by homo- or heterotetramers of six-transmembrane-segment subunits belonging to a vast family (K_V1.x-12.x, Gutman *et al*, 2005) of α subunits combining with an accessory β subunit. The S4 segment of each monomeric component contains several positively charged residues and forms part of the voltage sensor of the completed tetrameric protein (Figure 2a) which functions similarly to that of the VGSC. The P-loop of each monomer contains the consensus sequence TVGYG. Together in the assembled tetramer, the tyrosine and glycine residues along with nearby charged amino acids coordinate a single file of dehydrated K^+ ions, selectively allowing them to pass through the pore (Dibb *et al.*, 2003). Voltage-gated potassium channels activate in response to depolarization, but have slower activation than VGSCs, contributing to the delay in action potential repolarization. Their mechanisms of N-type and C-type inactivation are analogous to the fast and slow inactivation of VGSCs, respectively. The former involves a ball-and-chain mechanism in which the N-terminus of a monomeric domain occludes the inner face of the pore, and the latter involves conformational shifts in the S5-S6 loops (Figure 3b).

Cardiac potassium channels include the transient outward current $I_{to,s}$ and the delayed rectifier K^+ currents I_{Ks} and I_{Kr} found in atrial and ventricular cells as well as nodal tissue. $I_{to,f}$, $I_{K,slow}$ and the steady-state current I_{ss} are found in both ventricle and atrium but not in the nodes, while I_{Kp} and I_K are only in ventricle and I_{Kur} is only present

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in atrium (Nerbonne, 2000). These tissue distributions are based on studies in a wide range of species, however, and may not reflect the true distribution in the example of humans.

Delayed rectifier potassium currents are responsible for the repolarization of the action potential in ventricular and atrial myocytes. The major delayed rectifier currents are I_{Ks}, I_{Kr} and I_{Kur}, carried by $K_V7.1$, $K_V11.1$ and $K_V1.5$, respectively. Several cardiac channelopathies are associated with these potassium channels, including various forms of LQTS. Both LQT1 and LQT5 are linked to the KCNQ1 and KCNE1 genes encoding $K_V7.1$ (KvLQT1) and its accessory β subunit minK. LQT2 and LQT6 are both associated with the KCNH2 and KCNE2 genes encoding $K_V11.1$ (hERG) and its ancillary β subunit MiRP1 (Tamargo *et al.*, 2004). Mutations in potassium channel genes leading to these forms of LQTS are loss of function mutations, in which mutant channels have decreased potassium conductance, delaying repolarization, as opposed to the SCN5A mutations, which have a gain of function, whereby conductance is increased throughout the action potential.

Prolongation of the action potential by inhibiting delayed rectifiers is desired for the treatment of atrial fibrillation (AF), a relatively common tachyarrhythmia in which action potential duration and effective refractory period are decreased, possibly triggered by oxidative stress and decreased $I_{Ca,L}$ and perpetuated by atrial electrophysiological and structural remodeling (Korantzopoulos *et al.*, 2003).

Inward rectifier potassium currents include the strong inward rectifier current I_{K1}, whose conductance is high at potentials negative to E_K but is effectively inactive during the depolarized phases of the action potential due to block by intracellular cations such as

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Mg^{+} and polyamines. This permits a long action potential plateau and, together with high permeability of potassium relative to other ions, drives the resting membrane potential toward E_K (Dhamoon & Jalife, 2005).

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Figure 3. The voltage-gated potassium channel

a. Representation of the four six-transmembrane-segment subunits that form voltage-gated potassium channels. The S4 domain contains positively charged residues; the P-loop between S5 and S6 lines the pore; and the N-terminal sections are used in a mechanism of inactivation. **b.** Voltage-gated potassium channels open upon activation and deactivate to return to a closed state.

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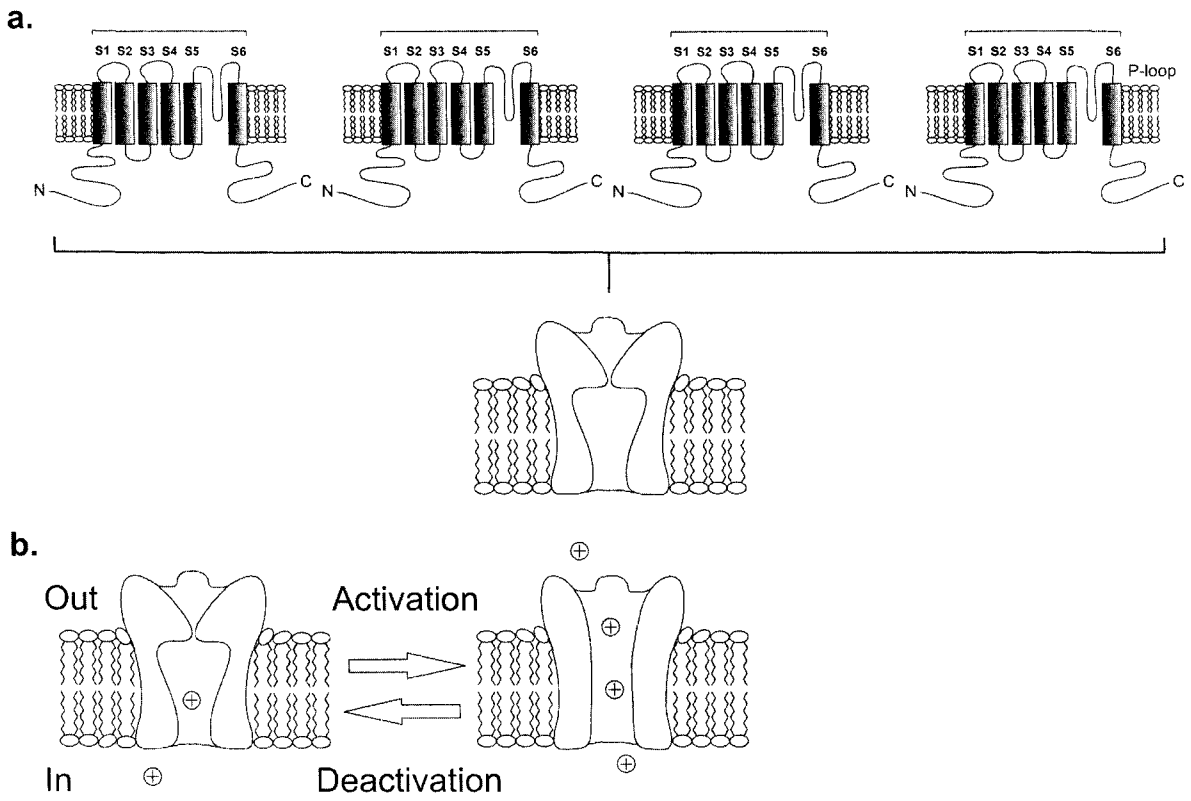

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Ischemic heart disease

Ischemic heart disease (IHD) is affecting an increasingly large proportion of the Western world, where the population is aging and lifestyle-related risk factors for cardiovascular disease are becoming more common. Recent prevalence statistics report that more than 13.2 million Americans have a form of IHD; 7.2 million suffered a myocardial infarction and 6.5 million have angina (American Heart Association *Heart Disease and Stroke Statistics* 2006 Update). Obesity, smoking, high blood cholesterol and blood pressure, diabetes and physical inactivity are all considered risk factors for IHD (Heart and Stroke Foundation of Canada *Heart Disease and Stroke in Canada*; Ottawa, 1997), and the prevalence of many of these has also been increasing in the past decade.

IHD comprises several clinical syndromes related to ischemia, an insufficiency of blood flow to the heart leading to necrosis of cardiac tissue accompanied by metabolic and electrophysiological changes. It is often caused by a restriction of coronary artery flow associated with atherosclerosis. Chronic artery stenosis may result in stable (reversible) angina or subendocardial infarction if there is a less than total occlusion. Heart failure is also a common correlate of ischemia as contractile tissue is compromised. Sudden cardiac death may arise due to ventricular arrhythmias caused by re-entrant circuits through electrophysiologically or anatomically remodeled tissue. If there is more severe but not necessarily widespread atherosclerosis involving plaque disruption and the formation of a thrombus, this may lead to unstable angina or transmural myocardial infarction. Other organs in the body, such as the kidneys, liver, GIT and/or brain may be secondarily affected by either a lack of blood supply due to heart failure or migration of a dislodged thrombus, as is the case in stroke.

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Treatment of these ischemic syndromes involves reperfusing blood to the heart, which may cause further damage as oxygen is reintroduced to cardiac tissue, inducing an inflammatory response as well as oxidative stress and ionic imbalances. The next section will focus on changes in the heart during ischemia-reperfusion (I/R) injury.

Cellular pathophysiology of ischemia-reperfusion injury

In healthy cardiac tissue, adequate perfusion provides cells with necessary resources such as oxygen and nutrients, and removes waste products such as carbon dioxide. Ischemia occurs when blood flow to the heart is severely restricted, therefore it (1) prevents aerobic metabolism, reducing [ATP]/[ADP][P_i] and permitting increased anaerobic metabolism, and (2) allows accumulation of the byproducts of both anaerobic and previous aerobic metabolism, including lactate, phosphates and carbon dioxide (Carmeliet, 1999). There are also increases in lipid metabolites and free radicals, which may contribute to cellular damage. Long-chain acyl carnitines, for example, inhibit the Na^+/K^+ATPase pump, which leads to disturbances in intracellular and extracellular ion concentrations (Carmeliet, 1999). The reduction in [ATP]/[ADP][P_i] decreases the free energy of ATP hydrolysis available to the Na^+/K^+ATPase, increasing its reversal potential from – 180 mV to – 60 mV. During ischemia, the cell membrane is depolarized to around – 60 mV, making the pump ineffective at the membrane potential. Further inhibition of the pump is contributed by free radicals produced during ischemia (Carmeliet, 1999).

Depolarization of the cell membrane is caused by inward leak, due to increased I_{NSC}, I_{Cl} and late I_{Na}, which will be discussed in the next section, and by an accumulation

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of extracellular potassium. $[K^+]_o$ is usually kept to ~ 4 mM but reaches up to 4 times its non-ischemic level within 30 minutes of ischemia (Carmeliet, 1999). In addition to decreased K^+ influx through the Na^+/K^+ATPase, the increase in $[K^+]_o$ is due to 1) shrinkage of the extracellular space when water enters the cell to compensate for increased concentrations of metabolites such as lactate and phosphates; 2) reduced washout and subsequent t-tubule accumulation and 3) increased conductance of various K^+ channel currents. These include I_{KATP}; I_{KNa}, activated by local increases in $[Na^+]_i$ due to increased late I_{Na} and Na^+/K^+ATPase inhibition; and I_{KAA}, activated by decreases in pH_i due to increased lactate and carbon dioxide. Depolarization also acts to promote K^+ efflux as E_m is kept away from E_K (Carmeliet, 1999).

Acidosis of both the extra- and intracellular spaces is an important feature of ischemia. Net proton production is increased due to lactate formation, ATP hydrolysis and retention of carbon dioxide, changing pH_i from 7.2 to ~ 6.5 (Carmeliet, 1999). Due to the reduction of extracellular perfusion, pH_o decreases from 7.4 to ~ 6.0 as protons build up during the course of ischemia (Carmeliet, 1999), also causing inhibition of the sodium hydrogen exchanger (NHE). Although the concomitant influx of sodium ions forms an important part of an increase of $[Na^+]_i$ at reperfusion, neither inhibition of NHE nor restoration of its function on reperfusion significantly alters pH levels. If the ischemic period is not too long, then reperfusion allows pH levels to rapidly normalize as lactate and carbon dioxide are washed out and energy is restored (Carmeliet, 1999).

Intracellular sodium is normally maintained at the 5-10 mM level, although subcellular concentrations may vary, with higher $[Na^+]$ in the subsarcolemmal space. During ischemia, $[Na^+]_i$ is elevated within 15-20 min to reach 20-25 mM due to

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inhibition of sodium extrusion via the Na^+/K^+-ATPase and increases in inward leak currents. Inward sodium flux occurs through 1) I_{NSC}, stimulated by stretch, $[ATP]_o$, $[Ca^{2+}]_i$ and free radical formation, all induced early in ischemia; 2) the partially inhibited NHE; and 3) VGSCs (Van Emous *et al.*, 1997), which may be altered by inhibition of the inactivation process during ischemia to carry a greater proportion of the late component of I_{Na}. The latter mechanism will be further discussed in the following section. This rise in $[Na^+]_i$ during ischemia, as well as a rapid transient rise in $[Na^+]_i$ as NHE activity is increased upon reperfusion, may activate 1) I_{KNa} potassium currents; 2) the Na^+/K^+-ATPase, causing a rapid normalization of $[Na^+]_i$ within 5 min of the end of a 30 min ischemic period; and 3) the reverse mode of the NCX, which brings in Ca^{2+}, contributing to calcium overload. All these mechanisms serve to repolarize the cell during reperfusion, reducing $[K^+]_o$ and shortening the action potential. Differences in the time course of repolarization between cell types within the myocardium may cause a dispersion of refractoriness, which, together with the overload of calcium, may lead to reperfusion-induced arrhythmias.

Many mechanisms contribute to abnormalities in calcium handling during I/R injury. Under healthy conditions, diastolic free $[Ca^{2+}]_i$ ranges from 50 – 200 nM. During systole, calcium is released from the SR, where calcium is stored at a level of ~700 μM, and from calcium-binding proteins, to reach 500 nM – 1 μM. Contraction ceases when intracellular calcium is again returned to stores. In the ischemic myocyte, diastolic free $[Ca^{2+}]_i$ increases after a 10 – 20 min delay and may exceed 1 μM, a diastolic level at which irreversible damage is likely. The rise is due to a number of factors, including inward Ca^{2+} leak via I_{NSC}; decreased forward mode NCX activity; increased reverse mode

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NCX activity due to elevated $[H^+]$, $[Na^+]$ and free radicals; decreased SR uptake due to loss of energy to drive the Ca^{2+}-ATPase; and protons competing out calcium ions in calcium-binding proteins. The latter mechanism as well as release from the SR may cause a transient rise in systolic $[Ca^{2+}]_i$, which is followed by a decrease. Mitochondria may become a source of excess Ca^{2+}, but are usually a sink during ischemia. Nevertheless, there is enough free Ca^{2+} to activate $I_{Cl(Ca)}$, I_{Ks} and I_{Kr} and inhibit $I_{Ca,L}$, contributing to action potential shortening during ischemia (Carmeliet, 1999). Upon reperfusion, there may be a rapid recovery to normal as the Ca^{2+}-ATPase and forward mode $I_{Na/Ca}$ are reactivated, but if $[Na^+]_i$ is still elevated, $[Ca^{2+}]_i$ may only return to an intermediate level. If ischemia has lasted longer than 20 min, diastolic $[Ca^{2+}]_i$ is likely greater than 1 μM, and excess calcium absorbed by mitochondria may cause irreversible damage. Elevated $[Ca^{2+}]_{mito}$, together with low [ATP] and oxidative stress during reperfusion, promote the opening of the mitochondrial permeability transition pore (mPTP), which leads to loss of mitochondrial electrochemical gradients, ATP hydrolysis, rigor, contracture and cell death (Carmeliet, 1999; Honda *et al.*, 2005). Alternatively, calcium overload may cause spontaneous calcium release from the SR during or after the action potential, leading to early or late afterdepolarizations, respectively.

Other important mechanisms that contribute to the electrophysiological changes in the I/R myocyte involve oxidative imbalances. Increased free radical, singlet oxygen and peroxide levels coupled with reduced antioxidative enzyme activity and free radical scavenger levels incur harmful oxidation of proteins and membrane lipids, directly and indirectly altering ion conduction. This leads to activation of SR I_{Ca}, I_{NSC}, I_{KATP} and opening of the mPTP and to inhibition of $I_{Ca(L)}$, fast I_{Na}, $I_{Na/K}$, $I_{Na/Ca}$ and other potassium

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currents (Carmeliet, 1999). The overall effect during ischemia occurs in two stages. 1) There is a decrease in action potential upstroke velocity due to I_{Na} block and depolarization; the latter also decreases the action potential amplitude; decreased outward and increased inward currents prolong the action potential duration; early and delayed afterdepolarizations may occur during this stage. 2) Opening of K_{ATP} channels produces a gradual shortening of the action potential, potentiated by mPTP opening, until a very short duration is reached, at which the diastolic potential is hyperpolarized to a point of cell inexcitability and hypercontracture (Carmeliet, 1999).

Late I_{Na} in ischemia-reperfusion injury

Late I_{Na} has been implicated in the ischemia portion of I/R injury (Xiao & Allen, 1999). While VGSCs normally inactivate within 10 ms and sodium entry is limited to the depolarization phase of the action potential, there are circumstances under which the inactivation process is dysfunctional, resulting in the development of a persistent current (late I_{Na}). In ischemia, these circumstances are still controversial, but they may involve cytoskeletal modulation (Undrovinas *et al.*, 1995); modification by accumulated lysophosphatidylcholine (Undrovinas *et al.*, 1992), due in part to increased long-chain acyl carnitine (Baetz *et al.*, 2003); a free radical-induced increase in the range of voltages over which sodium channels can open but not inactivate (Barrington *et al.*, 1997) and/or direct or indirect phosphorylation by PKA, PKC, AMPK and/or PTK or their pathways (Watson & Gold, 1997; Schreibmayer, 1999; Light *et al.*, 2003). High concentrations of intracellular sodium, accumulated through persistent VGSC activity, in addition to a depolarized membrane potential as discussed above, may cause the NCX to function in

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reverse mode, increasing levels of intracellular calcium ($[Ca^{2+}]_i$). This may cause the formation of premature afterdepolarizations and triggered activity, leading to ventricular arrhythmias as well as irreversible cell injury via calcium loading and subsequent hypercontracture (Carmeliet, 1999; Janse, 1999).

In summary, $[K^+]_o$ is increased during ischemia via excess K^+ efflux, reduced washout and Na^+/K^+-ATPase inhibition; this, as well as increased NHE activity due to lower pH_i and VGSC modification to produce a late I_{Na} cause an elevated $[Na^+]_i$; reverse mode NCX leads to a potentially damaging increase in and overload of $[Ca^{2+}]_i$., potentially leading to torsade de pointes and/or cell death. Reactive oxygen and nitrogen species play a role in the cellular damage caused by reperfusion injury and in the generation of arrhythmias. In conclusion, alterations in levels of K^+, H^+, Na^+ and Ca^{2+} due to changes in high-energy phosphate, lipid and oxidative metabolites during ischemia and reperfusion are fundamental to the changes observed in this pathophysiological state (Figure 4). Therefore agents that target ion channels and transporters to regulate levels of these ions and also reduce damage caused by ischemic byproducts will be very valuable against ischemic heart disease.

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Figure 4. Ionic changes in ischemia-reperfusion injury

a. Schema of ionic currents in an ischemic myocyte. (1): During ischemia, the [ATP]/[ADP][P_i] ratio is decreased and glycolysis is increased. (2): This leads to sodium-potassium pump inhibition and intracellular acidosis. The latter produces sodium influx via sodium-hydrogen exchange and these effects, in addition to increased persistent (late) sodium current lead to (3) elevated intracellular sodium. (4): High intracellular sodium and depolarization stimulate reverse-mode sodium-calcium exchange, (5) elevating intracellular calcium. (6): Calcium overload leads to severe myocardial damage.

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a.

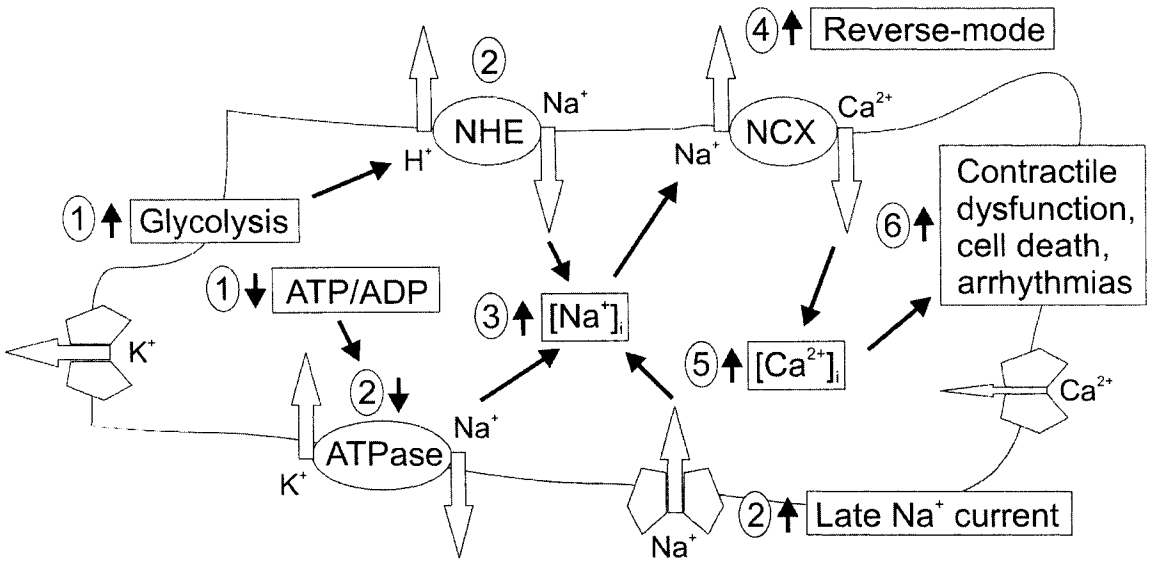

Figure 4.

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Long QT Syndrome channelopathies

Several human channelopathies, genetic disorders in which ion channel genes are responsible for pathophysiological states, involve late I_{Na}. Congenital mutations in the genes encoding VGSCs disrupt inactivation or uncouple activation from inactivation, causing such disorders as myotonia in skeletal muscle and epilepsy in the brain (George, 2005). In the heart, congenital LQT3 is caused by one of several mutations in the SCN5A gene encoding $Na_V1.5$, resulting in impaired inactivation of cardiac VGSCs, development of late I_{Na} and hence proarrhythmic prolonged action potential (Janse, 1999; Wang *et al.*, 1995). These mutations include R1644H and N1325S, found at the docking site of the inactivation loop, which destabilize the inactive state, increasing the rate of return to the open state, manifested as dispersed short single channel openings (Dumaine & Kirsch, 1998; Dumaine *et al.*, 1996). The ΔKPQ mutation is located in the inactivation loop itself and therefore also has characteristics of destabilized inactivation. There are also additional prolonged bursts of single channel openings due to a shift to a non-inactivating mode of gating (Bennett *et al.*, 1995; Dumaine & Kirsch, 1998; Clancy & Rudy, 1999). The R1623Q mutation is in the extracellular portion of the voltage sensor, and is thought to cause an uncoupling of activation and inactivation, leading to delayed inactivation and increased inactivation from the closed state. (Makita *et al.*, 1998; Kambouris *et al.*, 1998)

Acquired or drug-induced LQT3 is caused by polymorphisms in SCN5A that predispose to the LQT phenotype in the presence of a drug prolonging action potential duration. These mutations include R1193Q, a very common (0.2 % of a mostly caucasian population and 12 % of a Han Chinese population) single nucleotide polymorphism

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whose mechanism is similar to that of R1644H and N1325S (Wang *et al.*, 2004; Chen *et al.*, 2004).

The increased influx of sodium during the extended action potential plateau phase may promote the formation of early afterdepolarizations, and sodium overload may lead to calcium overload via reverse-mode $I_{Na/Ca}$, causing delayed afterdepolarizations. Both of these may lead to severe types of ventricular tachycardia such as torsade de pointes, which are maintained by reentry circuits based on differential rates of action potential repolarization in the epi-, endo- and midmyocardial subtypes of cardiac tissue (Janse, 1999; Antzelevitch, 2004).

VGSC inhibitors, including the class Ia and Ic antiarrhythmics lidocaine, flecainide and mexiletine, represent an important class of agents used to treat ventricular arrhythmias associated with LQT3. In the case of mutations leading to an uncoupling of VGSC activation and inactivation, blockers such as lidocaine increase the rate of transition to the inactivated state directly from the closed state, increasing the overall rate of inactivation (Kambouris *et al.*, 2000). For mutations in which the interaction between the inactivation loop and its receptor is dysfunctional, they counteract the increased rate of return from the inactivated state to the open state by binding to the inactivated state (Kambouris *et al.*, 1998).

Although class I antiarrhythmics have shown effectiveness in treating LQTS (Kambouris *et al.*, 1998) and their frequency-dependent block may be useful in targeting abnormal heart rate and thus avoiding conduction deficits in healthy tissue, reperfusion-induced arrhythmias have proven less amenable to these blockers (Farkas & Curtis, 2002). Identification of novel VGSC inhibitors that selectively inhibit late I_{Na} may

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provide useful pathology-specific pharmacological tools. The antianginal drug ranolazine shows such preferential inhibition, with block of both late I_{Na} and I_{Kr} in the low μM range, perhaps accounting for its observed ability to block *d*-sotalol-induced EADs (Antzelevitch *et al.*, 2004).

Atrial fibrillation

Atrial fibrillation (AF) is a relatively common tachyarrhythmia in which action potential duration (APD) and effective refractory period are decreased, possibly triggered by oxidative stress and decreased $I_{Ca,L}$ and perpetuated by atrial electrophysiological and structural remodeling (Korantzopoulos *et al.*, 2003).

Treatment for AF includes (1) restoring sinus rhythm via electrical or pharmacological cardioversion and preventing recurrence with further drug therapy; or (2) slowing the heart rate without promoting a return to sinus rhythm (Pacifico and Henry, 2003). Drugs for rate control include digoxin, used if CHF is present; VGCC blockers diltiazem and verapamil; and β-blockers metoprolol and esmolol. Drugs for rhythm conversion include class Ia and Ic VGSC blockers procainamide, quinidine, propafenone and flecainide; and class III potassium channel blockers sotalol, amiodarone, ibutilide and dofetilide. While drug therapy has not been shown to be more effective than electrical cardioversion, and in some cases may have more negative outcomes (Mittal *et al.*, 2000; Hopson *et al.*, 1996), the cost savings and ease of using antiarrhythmics is encouraging the search for better AF drugs, focusing especially on blockers selective for atrial ion channels. Class I and III antiarrhythmics have multiple ion channels as targets and may also affect metabolic pathways in the long term (Pacifico and Henry, 2003).

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Especially important in this regard is the proarrhythmic risk of blocking ventricular delayed rectifier potassium channels and thus prolonging the QT interval. Targeting these channels in the atrium alone, to achieve the desired increase in action potential duration without delaying ventricular repolarization, has thus become a focus for drug design (Pacifico and Henry, 2003).

I_{Kur} in atrial fibrillation

Several K^+ currents are responsible for the repolarization of the action potential in ventricular and atrial myocytes. Potassium channels formed by homotetramers of $K_V1.5$, which underlie the ultrarapid delayed rectifier potassium current I_{Kur}, are more highly expressed and seem to be functional only in atria (Fedida *et al.*, 2003). This provides a potential means for atrial-specific therapy for AF. While many previously characterized I_{Kur} blockers also block other channels, the compounds AVE 0118, S9947 and S20951 and the novel antiarrhythmic RSD 1235 are selective for I_{Kur} in a concentration-dependent manner and therefore show promise as atrial-specific potassium channel blockers (Brendel & Peukert, 2003; Knobloch *et al.*, 2004). Additional effects may be imparted by an increase the effective refractory period (Wirth & Knobloch, 2001).

Cardioprotection by dietary polyphenols

Bioactive compounds include vitamins and polyphenols (flavonoids, stilbenes, lignans and phenolic acids), found in several dietary plant sources (Manach *et al.*, 2004). They provide health benefits via antioxidant and other protective mechanisms against

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various forms of cancer as well as neurodegenerative and cardiovascular disease (Table 1).

Grape products, including red wine in particular, are important sources of several dietary polyphenols with concentrations estimated to range from 0.5 – 200 μg mL^{-1} for catechin and epicatechin; 0.25 – 50 μg mL^{-1} for quercetin; and 0.05 – 8.5 μg mL^{-1} for resveratrol (Bertelli *et al.*, 1998; Gambuti *et al.*, 2004; Leonard *et al.*, 2003; Manach *et al.*, 2004; Ray *et al.*, 1999). Other red wine polyphenols include myricetin, kaempferol, epicatechin-gallate, anthocyanins, caffeic acid, *p*-coumaric acid and ferulic acid (Leonard *et al.*, 2003). These polyphenolic constituents, especially the much-studied resveratrol, have demonstrated cardioprotective efficacy and are thought to be responsible for the well documented cardiovascular benefits of red wine and the 'French Paradox' of low mortality from cardiovascular disease among regular drinkers of moderate amounts of red wine (Renaud & de Lorgeril, 1992). Traditional Asian diets are also associated with a low incidence of cardiovascular illness. Green tea, which is often a large component of several of these diets, is another important source of cardioprotective monomeric flavanols, with catechin and epicatechin contents of 100 – 800 μg mL^{-1} (Manach *et al.*, 2004).

These polyphenols have exhibited a multitude of cardioprotective properties *in vitro* and *in vivo*, including anti-platelet, antioxidative, anti-ischemic and antiarrhythmic effects. In a model of I/R injury, EGCG infusion during reperfusion reduces myocardial damage (Aneja *et al*, 2004). This polyphenol has also been shown to be effective against evolving atherosclerotic lesions (Chyu *et al*, 2004), possibly via inhibition of smooth muscle cell proliferation (Hofmann & Sonenshein, 2003). Resveratrol, the best-

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characterized grape polyphenol, also benefits various cardiopathological situations. When resveratrol or red wine supplemented with resveratrol is added to healthy human plasma, both reduce platelet aggregation (Bertelli *et al*, 1995) and when compared to several other polyphenols and antioxidants, resveratrol and quercetin are effective inhibitors of thrombin- and ADP-induced platelet aggregation (Pace-Asciak *et al*, 1995). Resveratrol also directly inhibits the oxidation of human LDL (Frankel *et al*, 1993). In models of I/R injury, resveratrol improves functional recovery (Ray *et al*, 1999) and reduces both infarct size (Hung *et al*, 2000; Ray *et al*, 1999) and the severity of resultant ventricular arrhythmias (Hung *et al*, 2000). The reductions in I/R-induced cell damage may result from increased nitric oxide synthesis and the antioxidant effects of resveratrol but a separate, as yet uncharacterized mechanism may be involved, related to a reduction in I/R-induced arrhythmias (Hung *et al*, 2004), suggestive of an effect on cardiac ion channel function.

Ion channel modulation by dietary polyphenols

Polyphenols have long been recognized to contribute to human health through the diet. Their main benefits, against cancer and cardiovascular disease, have been attributed for the most part to their actions as free radical scavengers, with other health effects as secondary to these actions. However, in recent years, research has begun to focus more on their direct interactions with cell signaling molecules and ion channels. For instance, in cardiac cell lines, resveratrol has been shown to induce endogenous antioxidants and antioxidative enzymes (Cao & Li, 2004) and in a human endothelial cell line, resveratrol incubation stimulated Ca^{2+}-activated I_K independently of $[Ca^{2+}]_i$ (Li *et al*, 2000) and

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upregulated eNOS mRNA (Wallerath *et al*, 2002). Resveratrol and other phytoestrogens such as genistein and daidzein are thought to inhibit calcium influx into platelets via direct interaction with calcium channels (Dobrydneva *et al*, 2002) and resveratrol has been shown to inhibit peak I_{Na} in ganglionic neurons (Kim *et al*, 2005).

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Hypotheses and aims

The red grape polyphenols catechin, quercetin and resveratrol and the green tea polyphenol EGCG share the common structural feature of one or more phenolic ring(s) with VGSC-blocking drugs such as lidocaine (Figure 5). As the phenol group found in lidocaine is thought to impart late VGSC block (Haeseler *et al.*, 2002; Zamponi & French, 1993), it was hypothesized that polyphenolic compounds may also inhibit peak and/or late VGSC activity, contributing to the beneficial effects of these dietary polyphenols documented in settings such as I/R. In partial support of this hypothesis, it has recently been shown that resveratrol inhibits neuronal VGSCs (Kim *et al.*, 2005). The cardioprotective effects of polyphenols will be investigated via selective induction of late I_{Na} in myocytes to examine calcium handling and contractile dysfunction. In addition, it is hypothesized that these compounds may have inhibitory effects on other cardiac channels such as atrial $K_V1.5$ channels, implying potential therapeutic value in the treatment or prevention of atrial arrhythmias.

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Figure 5. Structural formulae

Quercetin **(a)**, catechin **(b)**, resveratrol **(c)** and epigallocatechin gallate **(d)** are antioxidant polyphenols with substituted aromatic structures. Lidocaine **(e)** is a VGSC blocker containing a similar aromatic group. N-acetylcysteine **(f)** is an antioxidant without structural similarity to these compounds.

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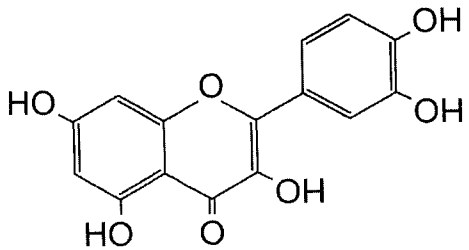
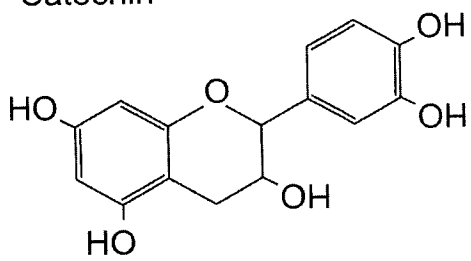
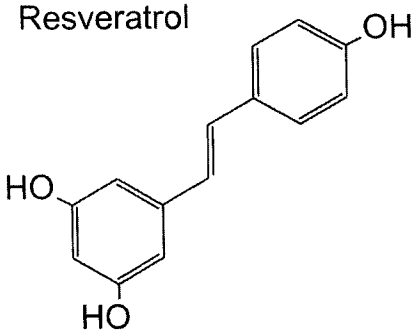
a. Quercetin



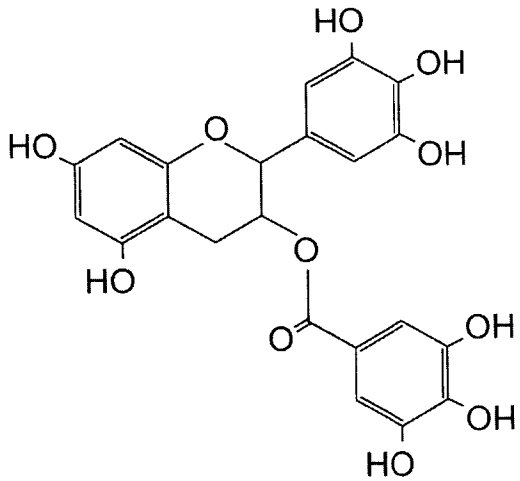
b. Catechin



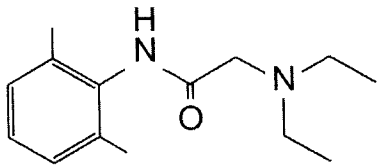
c. Resveratrol



d. Epigallocatechin gallate



e. Lidocaine



f. N-acetylcysteine

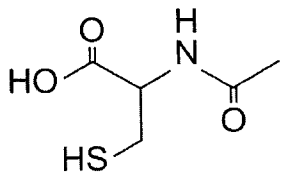


Figure 5.

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Table 1. Common polyphenols: dietary sources and health effects

Polyphenol	Type	Main dietary sources	Health effects	References
Resveratrol	Stilbene	Red wine (low concentration)	Prevents release of inflammatory mediators, inhibits platelet aggregation and LDL oxidation	Manach *et al.*, 2004; Rotondo *et al.*, 1998; Bertelli *et al.*, 1995; Brito *et al.*, 2002
Quercetin	Flavonol	Onions, kale, leeks, broccoli, blueberries	Inhibits platelet aggregation	Manach *et al.*, 2004; Pace-Asciak *et al.*, 1995
Catechin	Flavanol	Tea, chocolate, beans, fruit, wine	Prevents Aβ-induced neuronal cell death, inhibits angiogenesis and atherosclerosis	Manach *et al.*, 2004; Heo *et al.*, 2005; Chyu *et al*, 2004; Hofmann & Sonenshein, 2003
EGCG	Flavanol	Green tea, black tea	Reduces myocardial damage in I/R injury, inhibits atherosclerosis	Manach *et al.*, 2004; Aneja *et al.*, 2004; Chyu *et al.*, 2004

MATERIALS AND METHODS

Animal care

Adult male Sprague-Dawley rats (200-250 g) were used for experiments in accordance with guidelines set out by the University of Alberta Animal Policy and Welfare Committee and by the Canadian Council on Animal Care.

Cell culture and transfection

Human embryonic kidney tsA201 cells, a simian virus (SV-40)–transformed derivative of HEK-293 cells, were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10 % fetal calf serum and 0.1 % penicillin/streptomycin at 37 °C in 5 % CO_2. Passage numbers ranging from 20 to 60 were used. Cells were plated at 50 % to 70 % confluency onto 35 mm culture dishes 3 to 4 hours before transfection. Mammalian expression vectors encoding either the rabbit portal vein ultra-rapid delayed rectifier potassium channel clone $K_V1.5$ or the human heart VGSC clone $Na_V1.5$—either wild-type or with the R1623Q mutation (see below)—were cotransfected with vectors for green fluorescent protein (GFP) (pGL, Life Technologies, Burlington, ON, Canada) as a visual marker into cells using the calcium phosphate precipitation technique. $Na_V1.5$ was generously provided by Dr. A. M. Brown (Case Western Reserve University, Cleveland, OH, U.S.A.) and $K_V1.5$ was generously provided by Dr. W. C. Cole (University of Calgary, Calgary, AB, Canada). Cells were plated at 10 % to 30 % confluency onto cover slips 40 to 45 h after transfection. Single cells showing robust GFP expression were used for electrophysiological recording during

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the subsequent 30-h period. For experiments using the rat sodium-calcium exchanger (NCX1.1), tsA201 cells were infected with 30 pfu $cell^{-1}$ of an NCX1.1 construct in an adenovirus construct, generously provided by Dr. J. Lytton (University of Calgary, Calgary, AB, Canada) and Dr. J. Y. Cheung (Geisinger Medical Center, Danville, PA, U.S.A.).

Site-directed Mutagenesis

An amino acid mutation was created in the $Na_V1.5$ α subunit substituting an arginine residue with a glutamine at position 1623 (R1623Q) using the polymerase chain reaction. A 575 bp cDNA of $Na_V1.5$ was amplified using oligonucleotide primers "1623SeqF" (5′-A_{4305}GAGCAGCCTCAGTGGGA-3′) and "1623L" (5′-G_{4879}GCGGATGACTTGGAAGA-3′) (Operon Biotechnologies, Inc., Germantown, MD, U.S.A.). At the same time, a 474 bp cDNA of $Na_V1.5$ was amplified using the primers "1623U" (5′-G_{4857}ACGCTCTTCCAAGTCAT-3′) and "1623SeqR" (5′-A_{5330}CGCTGAAGTTCTCCAGGA-3′). The pairs of PCR products were purified via gel-extraction and combined in a second round of PCR with the primer pair "1623SeqF" and "1623SeqR", giving 1026 bp fragments. The resulting PCR products were digested with *Bsr*GI to yield 964 bp inserts, which were then subcloned back into wild-type $Na_V1.5$ to produce a construct confirmed by sequencing.

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Electrophysiology

Pipettes were pulled from borosilicate glass capillary tubing (Warner instruments, Hamden, CT, U.S.A.) using a P-87 micropipette puller (Sutter Instruments, Novato, CA, U.S.A.) and the tips were fire-polished, yielding resistances of 1-4 MΩ.

The pipette solution used for the measurement of $Na_V1.5$ current contained (in mM): 130 CsCl, 5 NaCl, 5 TEA-Cl, 2.5 HEPES, and 1 EGTA. The pH was adjusted to 7.2 with CsOH. 2 mM MgATP was added immediately before use. Cells were bathed in extracellular solution containing (in mM): 140 NaCl, 10 HEPES, 1 $CaCl_2$, 1.4 $MgCl_2$, 5 KCl, and 10 glucose (pH adjusted to 7.4 with NaOH). Solutions (see below) were applied to cells using a multi-input perfusion pipette. Whole-cell voltage-clamp was used to record macroscopic $Na_V1.5$ current. Data were recorded using an Axopatch 200B patch-clamp amplifier, compensating for capacitance and series resistance, and Clampex 8 software (Axon Instruments, Foster City, CA, U.S.A.). Test pulses to – 20 mV from a resting potential of – 100 mV lasted 80 ms with a cycle length of 0.2 Hz or 20 ms at 10 Hz. When appropriate, late I_{Na} was measured 50 ms after initiation of the test pulse. Steps of 40 ms from – 100 mV to between – 100 mV and + 90 mV in 10 mV increments were also recorded and data fitted to the Boltzmann equation (see below) to yield g_{max} curves for voltage-dependence analysis. To examine steady-state availability from inactivation, a protocol consisting of a 500 ms prepulse step from – 100 mV to + 20 mV followed by 20 ms steps to between – 100 mV and – 60 mV in 5 mV increments and a final 20 ms step to 0 mV was used. Due to space-clamp concerns, data were discarded if the resulting slope factor was < 6 mV.

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Measurement of $K_V1.5$ current was performed using a pipette solution containing (in mM): 10 KCl, 130 Aspartic Acid, 1.4 $MgCl_2$, 10 HEPES, and 1 EGTA. The pH was adjusted to 7.2 with KOH. 2 mM MgATP was added immediately before use. The whole-cell voltage-clamp technique was used as above and cells were again bathed in extracellular solution and experimental compounds (see below) applied using a multi-input pipette. Test pulses to 40 mV from a resting potential of – 80 mV lasted 100 ms with a cycle length of 0.2 Hz.

NCX1.1 current measurements were obtained using the inside-out patch-clamp technique. Outward reverse-mode currents were elicited by rapidly switching from a cesium-based intracellular solution containing (in mM): 120 CsCl, 20 TEA, 5 HEPES, 10 Glucose, 2 MgATP, 1.4 $MgCl_2$, 4.28 $CaCl_2$, and 5 EGTA to a sodium-based intracellular solution containing (in mM): 30 CsCl, 90 NaCl, 20 TEA-Cl, 5 HEPES, 10 glucose, 2 MgATP, 1.4 $MgCl_2$, 4.28 $CaCl_2$, and 5 EGTA for a duration of 50 s. Solutions (see below) were applied using a multi-input perfusion pipette. The pH of these solutions was adjusted to 7.2 with CsOH.

Myocyte isolation

Rats were euthanized with pentobarbital (150 mg kg^{-1}, i.p.) according to University of Alberta Animal Policy and Welfare Committee and Canadian Council on Animal Care Guidelines. The hearts were then removed, and the aortas were cannulated on a Langendorff apparatus and perfused with a solution containing 1 mM $CaCl_2$ for 5 min, followed by a solution containing 5 μM $CaCl_2$ for 7-10 min. This was followed by an enzyme solution containing 0.013 mg/ml collagenase (Yakult Honsha, Tokyo, Japan),

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0.013 mg/ml protease (type XIV; Sigma, St. Louis, MO) and 40 μM $CaCl_2$. After 10-15 min, the free wall of the right ventricle was removed and cut into smaller pieces for further incubation (in a shaker bath at 37°C) in a solution containing 0.08 mg/ml collagenase, 0.08 mg/ml protease, 3 mg/ml bovine serum albumin (BSA), and 0.1 mM $CaCl_2$. Cells were stored in an enzyme-free solution containing 0.1 mM $CaCl_2$ and 3 mg/ml BSA, and aliquots were taken during the next 1 h to be placed on coverslips for observation at ×200 with a CK40 inverted microscope (Olympus, Melville, NY, U.S.A.) and superfused with control solution containing (in mM): 140 NaCl, 10 HEPES, 2.0 $CaCl_2$, 1.4 $MgCl_2$, 5 KCl, and 10 glucose.

Measurement of calcium transients

Right ventricular myocytes were loaded with 4 μM of the calcium-sensitive fluorescent probe Calcium Green-1AM (Molecular Probes, Eugene, OR, U.S.A.) using Pluronic F-127 (Molecular Probes, Eugene, OR, U.S.A.) to aid solubilization, for 30 min at room temperature followed by 30 min at 37°C. After loading, cells were washed with control solution and placed on coverslips for observation at ×200. Cells were then superfused with either control solution as described above, or experimental solutions described below, and field-stimulated at 1 Hz with 2 ms square pulses at a constant current 20% above threshold value. A Photon Technology International Photomultiplier Detection System (PTI, Lawrenceville, NJ, U.S.A.) and Clampex 8 software were used for data acquisition. Calcium Green-1AM was excited with light at 480 nm, and the emitted light intensity at 520 nm was digitized. Diastolic calcium was normalized to the peak amplitude of the calcium transient and measured as a percentage of control.

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Measurement of cell shortening

Right ventricular myocytes were washed with control solution and placed on coverslips for observation at ×200 with a CK40 inverted microscope. Cells were then superfused with either control solution as described above, or experimental solutions described below, and field-stimulated at 1 Hz with 2 ms square pulses at a constant current 20 % above threshold value. Clampex 8 software was used for data acquisition. Cell shortening was measured using a video edge detector (Crescent Electronics, Salt Lake City, UT, U.S.A.) and expressed as fractional change in cell length ($\Delta L = (L_0 - L)$ L_0^{-1}, where L is length upon stimulation and L_0 is resting cell length).

All experiments were performed at room temperature (21 ± 1 °C).

Experimental compounds

Grape extract (BioVin, Cyvex Nutrition, Inc., CA, U.S.A.) was prepared as a 15 mg mL^{-1} stock solution in dimethyl sulphoxide. All other drugs used in this study were obtained from Sigma (St. Louis, MO, U.S.A.) and were also prepared as stock solutions in dimethyl sulphoxide: Resveratrol at 10, 20, 50, 100 and 200 mM; quercetin at 1, 2, 5, 10, 20 and 50 mM; catechin at 10, 20, 50, 100 and 200 mM; EGCG at 50 mM; lidocaine at 50 mM; N-acetylcysteine at 200 mM and the VGSC inactivation inhibitor *Anemonia sulcata* toxin (ATX) II, used to induce a late I_{Na} (Chahine *et al.*, 1996), at 3 or 5 μM. Each stock solution was diluted 1000-fold in extracellular bath solution directly before use, to yield micromolar or nanomolar concentrations (Table 2). Dimethyl sulphoxide (0.1 % v v^{-1}) was used in control solutions.

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Data analysis and statistics

Data were analysed using Clampex 8, Microsoft Excel and Origin Graph. Data are presented as mean ± s.e.mean or as a fit to the Boltzmann equation.

For voltage-dependence curves:

Normalized conductance = $g(V)/g_{max} = 1+\exp((V-V_{1/2})/s)$,

where $g(V)$ is the conductance, g_{max} is the maximal conductance, V is the voltage, $V_{1/2}$ is the half-maximal voltage and s is the slope factor, zF/RT

For concentration-response curves:

Normalized current = $I/I_{max} = 1/(1+([]/IC_{50})^n)$,

where I is the current, I_{max} is the maximal current, $[]$ is the concentration, IC_{50} is the concentration producing half-maximal current inhibition and n is the hill coefficient.

Statistical analyses of data were performed using the Student's paired or unpaired *t* test or ANOVA as appropriate. A P value < 0.05 was considered statistically significant.

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Table 2. Experimental compounds: concentrations and sources

Experimental compound	Stock concentration(s)	Final concentration(s)	Source
Grape extract	15 mg mL^{-1}	15 µg mL^{-1}	Cyvex Nutrition (U.S.A.)
Resveratrol	10, 20, 50, 100 and 200 mM	10, 20, 50, 100 and 200 µM	Sigma (St. Louis, U.S.A.)
Quercetin	1, 2, 5, 10, 20 and 50 mM	1, 2, 5, 10, 20 and 50 µM	Sigma (St. Louis, U.S.A.)
Catechin	10, 20, 50, 100 and 200 mM	10, 20, 50, 100 and 200 µM	Sigma (St. Louis, U.S.A.)
EGCG	50 mM	50 µM	Sigma (St. Louis, U.S.A.)
Lidocaine	50 mM	50 µM	Sigma (St. Louis, U.S.A.)
N-Acetylcysteine	200 mM	200 µM	Sigma (St. Louis, U.S.A.)
Anemonia sulcata toxin (ATX) II	3 or 5 µM	3 or 5 nM	Sigma (St. Louis, U.S.A.)
Dimethyl sulphoxide	0.1 % v v^{-1}	0.0001 % v v^{-1}	Sigma (St. Louis, U.S.A.)

RESULTS

Grape extract: $Na_V1.5$ and $K_V1.5$ block

In order to first assess the VGSC and potassium channel blocking properties of grape-derived polyphenols, the effects of grape extract on peak $Na_V1.5$ and $K_V1.5$ currents were tested. Application of 15 μg mL^{-1} grape extract significantly inhibited $Na_V1.5$ (peak I_{Na}) to 47.0 ± 6.50 % of control (Figure 6b) but had less inhibitory effect on $K_V1.5$, diminishing current to 75.6 ± 5.87 % of control (Figure 6d). Washout of grape extract did not occur when perfusion ceased in cells expressing either channel.

Polyphenols: $Na_V1.5$ and $K_V1.5$ block

Since grape extract and red wine contain a variety of bioactive polyphenolic compounds, the effects of three of the most commonly occurring polyphenols, catechin, quercetin and resveratrol, as well as the green tea polyphenol epigallocatechin gallate (EGCG) were tested individually on recombinant $Na_V1.5$ and $K_V1.5$ (Figure 7). Concentration-dependence studies yielded IC_{50}s for the effects of resveratrol, quercetin and catechin on $Na_V1.5$ current of 77.3 ± 8.20 μM, 19.4 ± 2.05 μM and 76.8 ± 5.15 μM respectively (Figure 7e). 50 μM EGCG inhibited $K_V1.5$ current to 81.7 ± 1.95 % of control and resveratrol inhibited $K_V1.5$ current with an IC_{50} of 40.0 ± 6.80 μM (Figure 7h). To determine whether the VGSC blocking effects of the polyphenols could be attributed to the documented antioxidant properties of these compounds, the effects of the structurally unrelated antioxidant N-acetylcysteine (200 μM) was tested in parallel with

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the polyphenolic antioxidants. It was found to have no significant effect on $Na_V1.5$ current (Figure 7e).

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Figure 6. Grape extract: **$Na_V1.5$ and $K_V1.5$** block

a. Representative $Na_V1.5$ current traces in the presence of 0 or 15 μg mL^{-1} grape extract. **Inset.** Square voltage pulse representing the protocol used to induce $Na_V1.5$ current. **b.** Grape extract (15 μg mL^{-1}, n = 4, $P < 0.05$) inhibited Na1.5 current (peak I_{Na}). **c.** Representative $K_V1.5$ current traces in the presence of 0 or 15 μg mL^{-1} grape extract. **Inset.** Square voltage pulse representing the protocol used to induce $K_V1.5$ current. **d.** Grape extract (15 μg mL^{-1}, n = 4, $P < 0.05$) inhibited $K_V1.5$ current.

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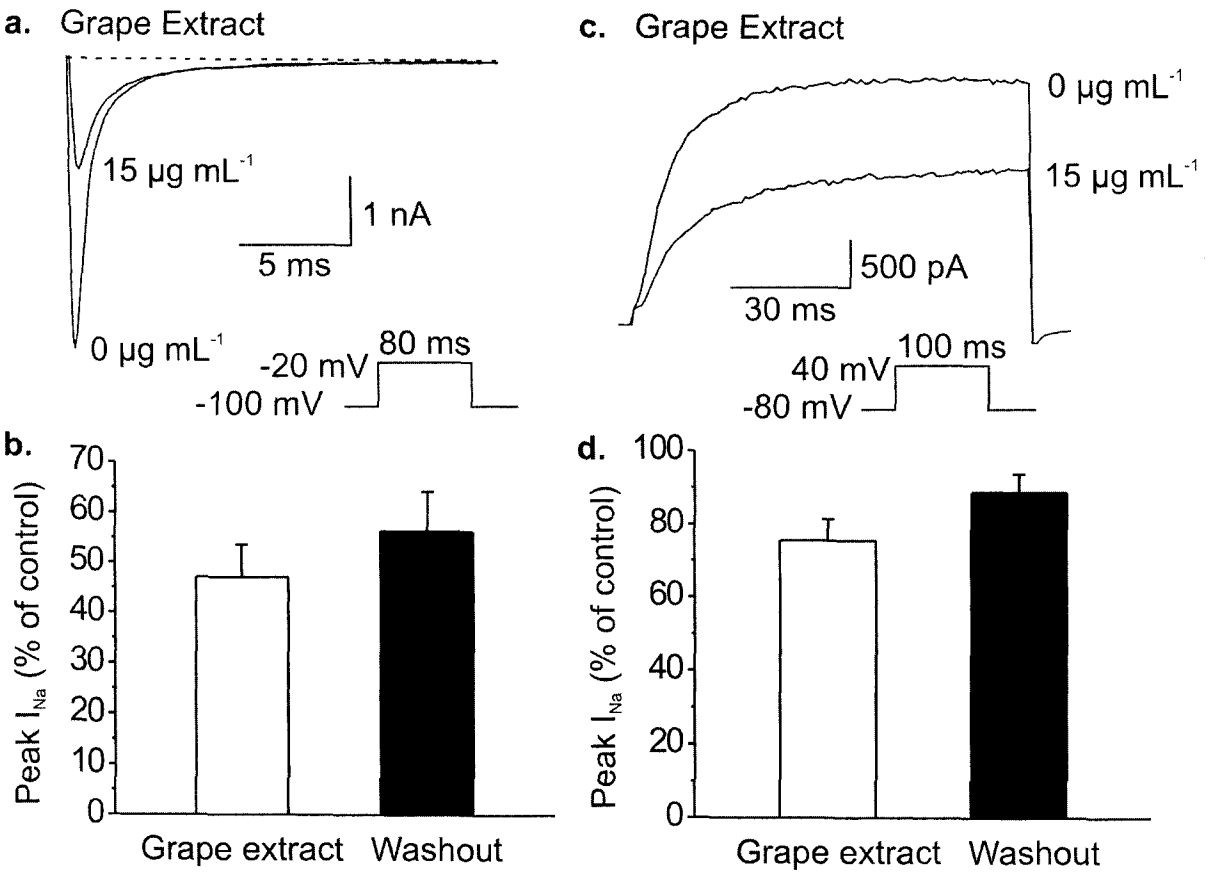

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Figure 7. Polyphenols: $Na_V1.5$ and $K_V1.5$ block

a. Representative $Na_V1.5$ current traces in the presence of 0, 10, 20, 50 or 100 μM resveratrol. **b.** Representative $Na_V1.5$ current traces in the presence of 0, 1, 10, 20 or 50 μM quercetin. **c.** Representative $Na_V1.5$ current traces in the presence of 0, 10, 20, 50 or 200 μM catechin. **d.** Representative traces of $Na_V1.5$ current in the presence of 200 μM N-acetylcysteine. **e.** Concentration-response curves for the effects of dietary polyphenols on $Na_V1.5$ current. Resveratrol, catechin and quercetin block peak I_{Na} (n = 4–11). 200 μM N-acetylcysteine does not (n = 7). $P > 0.05$. **f.** Representative $K_V1.5$ current traces in the presence of 0, 10, 20, 50 or 100 μM resveratrol. **g.** Representative traces of $K_V1.5$ current in the presence of 0 or 50 μM EGCG. **h.** Concentration-response curves for the effects of dietary polyphenols on $K_V1.5$ current. Resveratrol (n = 6) and EGCG (n = 3, $P < 0.001$) inhibit $K_V1.5$ current.

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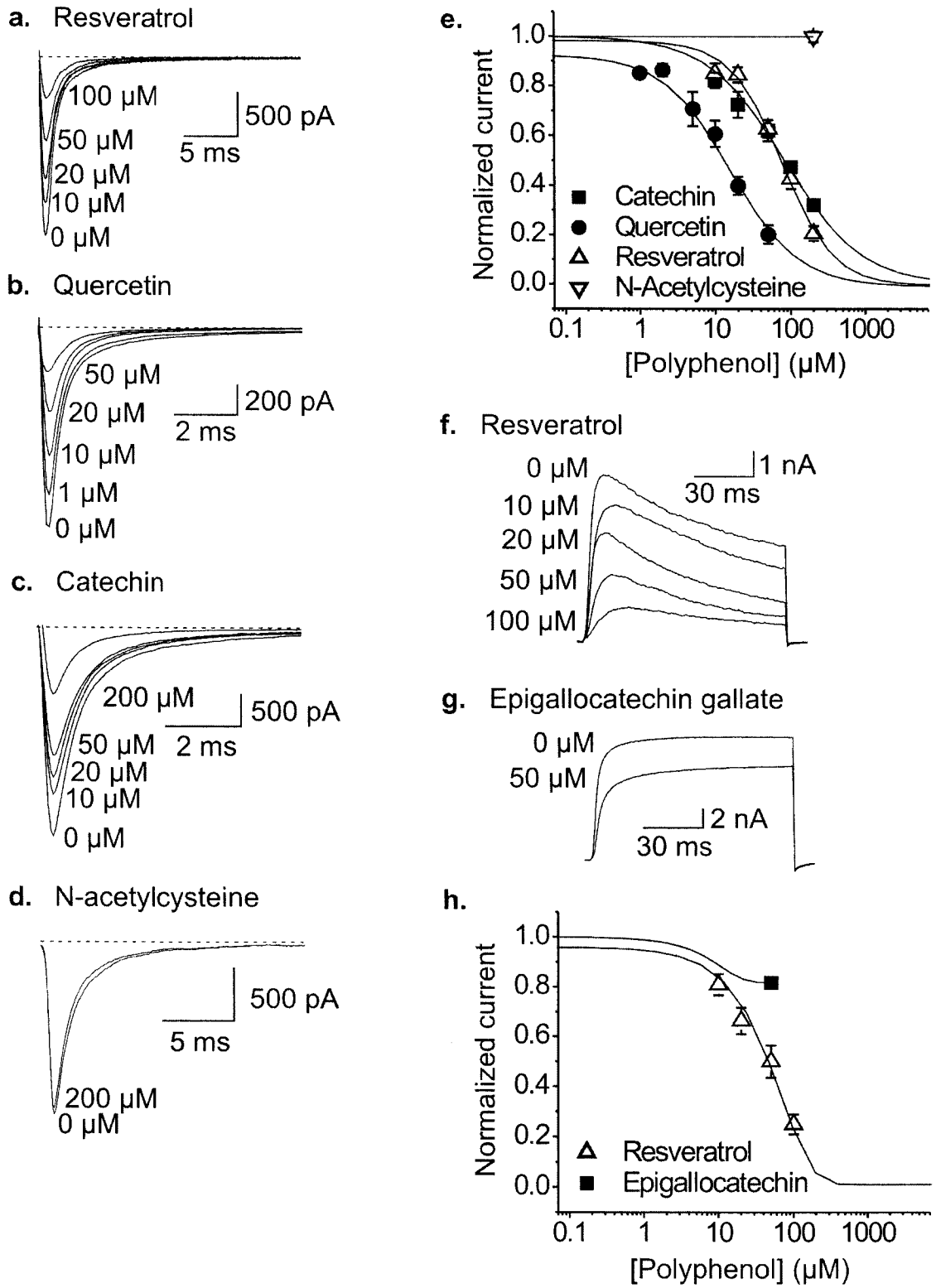

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Peak I_{Na} block: biophysical parameters

To further characterize peak I_{Na} block by polyphenols, other parameters such as the voltage-dependence of VGSC conductance and steady-state availability from inactivation were evaluated. $V_{1/2}$ in the presence of 50 μM resveratrol (-48.7 ± 0.942 mV) or 10 μM quercetin (-43.3 ± 0.498mV) was not significantly different from control (-48.1 ± 1.40 mV) (Figure 8a); nor was steady-state availability, for which $V_{1/2}$ was -93.2 ± 0.596 mV for control, -96.6 ± 0.936 mV for quercetin and -96.7 ± 1.21 mV for resveratrol (Figure 8b).

Peak I_{Na} block: frequency-dependence

One of the key properties of the antiarrhythmic agent lidocaine is the use- or frequency-dependence of its VGSC block. The frequency-dependence of resveratrol's $Na_V1.5$ current block was thus compared to that of lidocaine. At the higher stimulation frequency of 10 Hz, resveratrol and lidocaine (50 μM) inhibited peak I_{Na} by 38.3 ± 7.12 % and 66.8 ± 3.51 % respectively (Figure 9c). In constrast, at a lower pulse frequency of 0.2 Hz, resveratrol and lidocaine (50 μM) inhibited peak I_{Na} by 35.8 ± 3.48 % and 37.5 ± 6.04 % (Figure 9c).

Peak I_{Na} block: washout

The extent of washout at 0.2 Hz in the previous experiments was analyzed. The effect of lidocaine was fully reversible (101.3 ± 1.72 %) while the effect of resveratrol (50 μM) on peak I_{Na} was not (71.7 ± 3.70 % of control $Na_V1.5$ current, Figure 9d).

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Figure 8. Peak I_{Na} block: biophysical parameters

a. The voltage-dependence of VGSC conductance at varying test potentials. $V_{1/2}$ is unchanged with 50 μM resveratrol or 10 μM quercetin present (n = 5–10). **b.** The voltage-dependence of steady-state availability from VGSC inactivation ($V_{1/2}$) at varying prepulse potentials is unchanged with 50 μM resveratrol or 10 μM quercetin present (n = 5).

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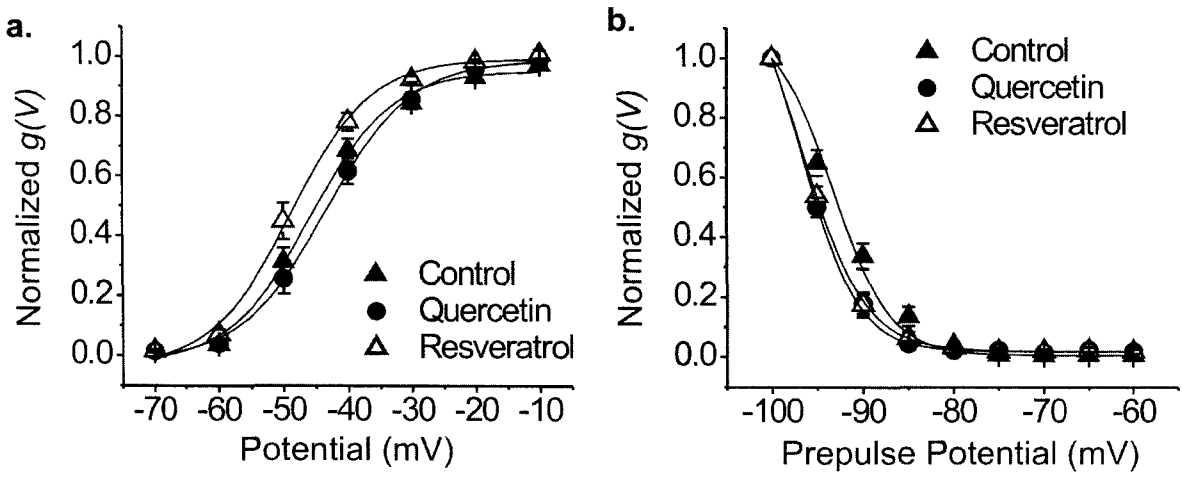

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Figure 9. Peak I_{Na} block: frequency-dependence and washout

a. Representative time-course of peak I_{Na} before, during and after application of 50 μM resveratrol at pulse frequencies of 0.2 Hz or 10 Hz. **Inset.** Representative $Na_V1.5$ current traces in the absence and presence of 50 μM resveratrol at 10 Hz. **b.** Representative time-course of peak I_{Na} before, during and after application of 50 μM lidocaine at pulse frequencies of 0.2 Hz or 10 Hz. **Inset.** Representative $Na_V1.5$ current traces in the absence and presence of 50 μM lidocaine at 10 Hz. **c.** At a pulse frequency of 0.2 Hz, resveratrol and lidocaine, at 50 μM each, inhibit $Na_V1.5$ current to a similar extent (n = 6–7). At a pulse frequency of 10 Hz, lidocaine (50 μM, n = 7) inhibits $Na_V1.5$ current to a greater extent than resveratrol (50 μM, n = 7). **d.** Peak I_{Na} blocked by 50 μM lidocaine at 0.2 Hz returns to control levels upon washout; peak I_{Na} block by 50 μM resveratrol at 0.2 Hz shows no significant washout (n = 6). $^{\#}P < 0.05$ within groups; $^{*}P < 0.05$ between groups.

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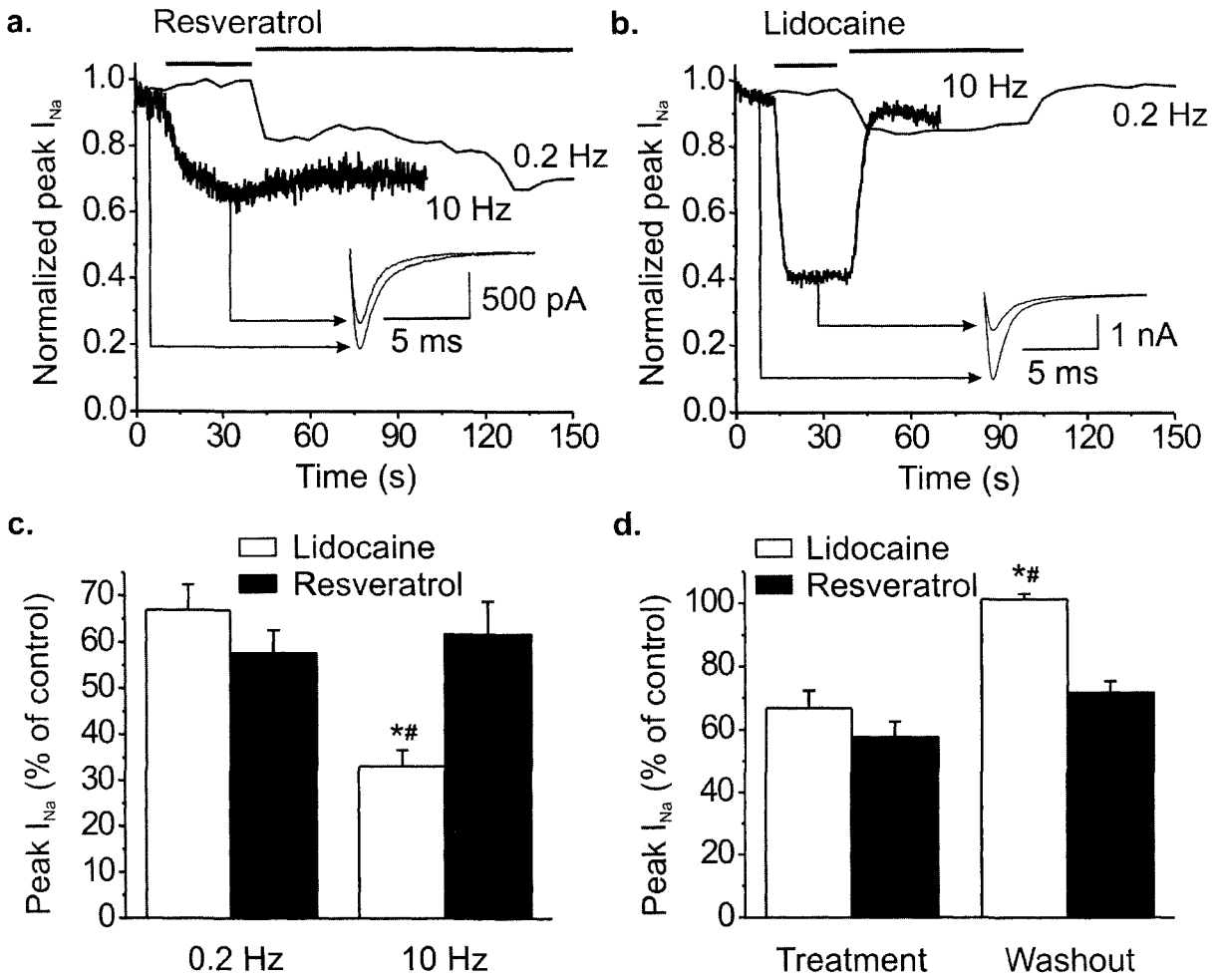

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Late I_{Na} block: mutant VGSC

The induction of late non-inactivating I_{Na} has been suggested to contribute to the ionic disturbances and consequent electrical and contractile dysfunction observed during I/R injury (Xiao & Allen, 1999). In addition, mutations in VGSCs found in LQT3 lead to pro-arrhythmic increases in APD by increasing non-inactivating late I_{Na}. As inhibition of late VGSC activity has the potential to be cardioprotective and antiarrhythmic, the effects of resveratrol and quercetin on mutant $Na_V1.5$ channels containing the LQT3 mutant R1623Q (Figure 10) were tested. Resveratrol (50 μM) exhibited a higher efficacy for late I_{Na} block in the R1623Q LQT3 $Na_V1.5$ mutant, reducing peak and late R1623Q I_{Na} to 67.7 ± 1.81 % and 28.9 ± 4.96 % of control values respectively (Figure 10d). In contrast, quercetin (10 μM) showed no selectivity between peak and late R1623Q I_{Na}, which were inhibited to 77.9 ± 4.02 % and 74.2 ± 9.44 % of control respectively (Figure 10d).

Late I_{Na} block: ATX II

In order to further test the effects of resveratrol and quercetin on late I_{Na}, ATXII (5 nM) was used to selectively induce a non-inactivating late I_{Na} (Chahine *et al.*, 1996) in tsA201 cells expressing wild type $Na_V1.5$ (Figure 11a, inset). At 50 ms after initiation of the depolarizing pulse, ATXII induced a 20-fold increase in late I_{Na} that was inhibited by resveratrol with an IC_{50} of 26.1 ± 2.93 μM (Figure 11b). This block was 3-fold greater than inhibition of peak I_{Na} (IC_{50} of 77.3 ± 8.20 μM) (Figure 11b). Quercetin showed similar inhibition of peak and late I_{Na}, with IC_{50}s of 13.6 ± 2.17 μM and 24.9 ± 3.23 μM respectively (Figure 11d).

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Figure 10. Late I_{Na} block: mutant VGSC

a. Normalized traces comparing currents from mutant and wild-type $Na_V1.5$ channels. **b.** Representative traces of $Na_V1.5$ current through mutant (R1623Q) VGSCs in the absence and presence of 50 μM resveratrol. Measurements of late I_{Na} were taken at a time-point 50 ms after initiation of the depolarizing pulse. **c.** Representative traces of $Na_V1.5$ current through mutant (R1623Q) VGSCs in the absence and presence of 10 μM quercetin. **d.** Peak and late I_{Na} through mutant (R1623Q) VGSCs in the presence of 50 μM resveratrol or 10 μM quercetin. All groups were normalized to their controls. Peak R1623Q I_{Na} was blocked to a greater extent than late I_{Na} during application of 50 μM resveratrol (n = 7) and peak R1623Q I_{Na} was blocked to the same extent as late I_{Na} during application of 10 μM quercetin (n = 10). $P < 0.001$. **Site-directed mutagenesis performed by Matthew Fercho.**

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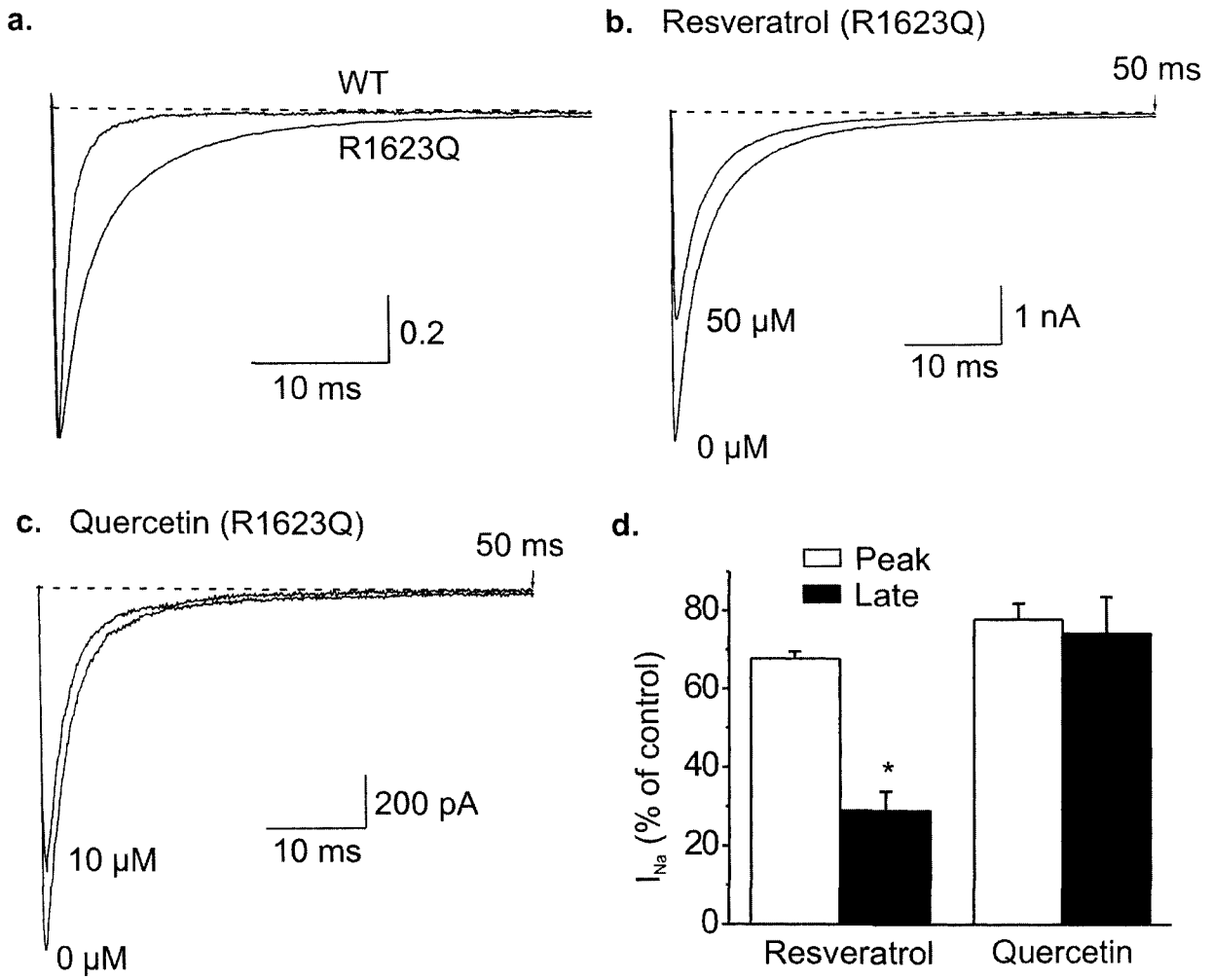

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Figure 11. Late I_{Na} block: ATX II

a. Representative $Na_V1.5$ current traces in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM resveratrol. **Left inset.** Representative $Na_V1.5$ current traces in the presence of control solution and 5 nM ATXII. **Right inset.** Expanded trace of late I_{Na} in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM resveratrol 40–60 ms after the depolarizing pulse. Measurements of late I_{Na} were taken at a time-point 50 ms after initiation of the depolarizing pulse. **b.** Concentration-response curves for the effects of resveratrol on $Na_V1.5$ current. Resveratrol blocks ATXII-induced late I_{Na} to a greater extent than peak I_{Na} (n = 5–11). **c.** Representative $Na_V1.5$ current traces in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM quercetin. **Inset.** Expanded trace of late I_{Na} in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM quercetin 40–60 ms after the depolarizing pulse. Measurements of late I_{Na} were taken at a time-point 50 ms after initiation of the depolarizing pulse. **d.** Concentration-response curves for the block of peak I_{Na} and ATXII-induced late I_{Na} by quercetin. Quercetin shows similar inhibition of peak and late I_{Na} (n = 4).

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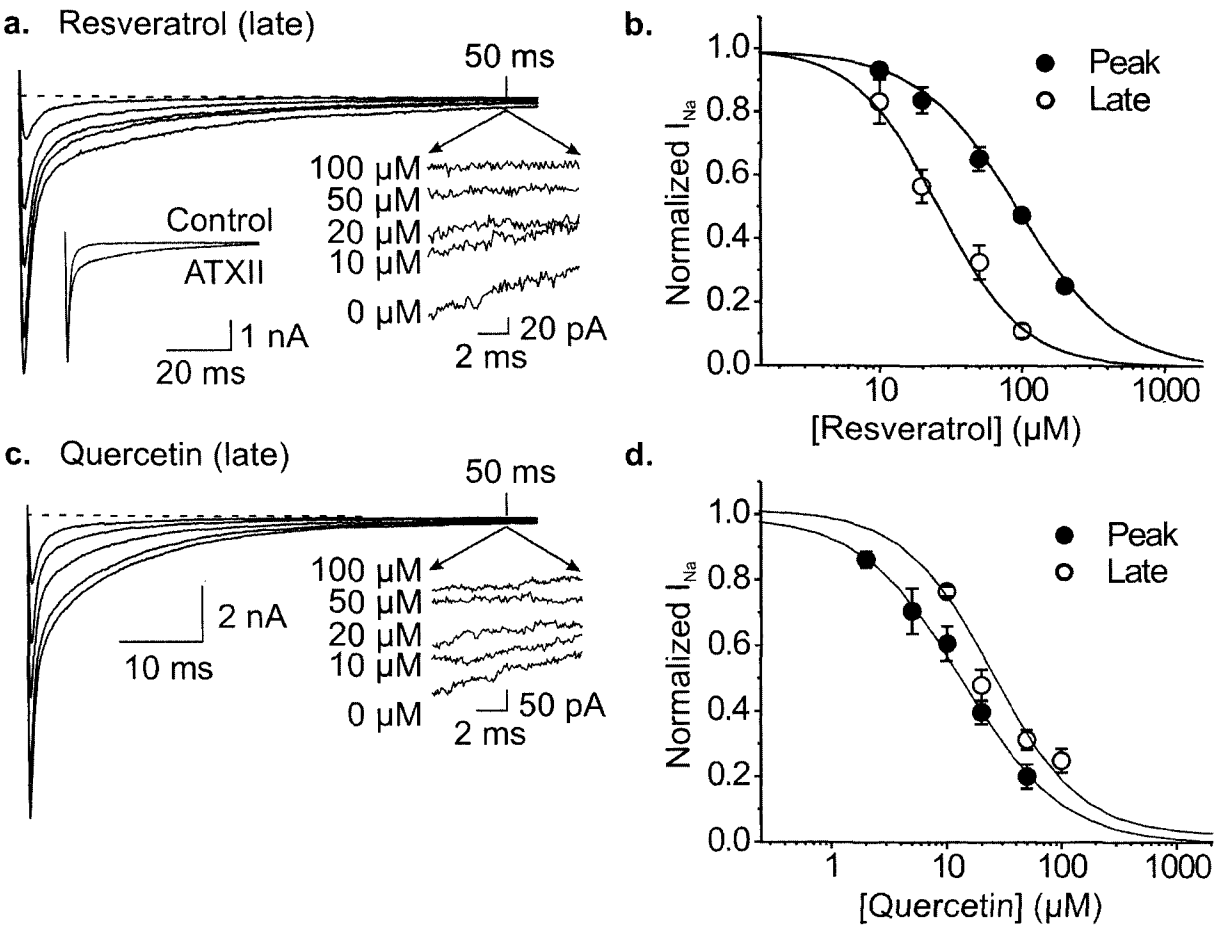

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Myocyte calcium transients

The studies of resveratrol on recombinant $Na_V1.5$ channels suggest that inhibition of sodium current, particularly late I_{Na}, may be involved in some of its documented cardioprotective effects, due to a subsequent reduction in damaging calcium overload. In order to test the ability of resveratrol to reduce myocardial dysfunction, ATXII was used in isolated rat right ventricular myocytes to specifically induce late I_{Na} (Chahine *et al.*, 1996) and hence alterations in calcium homeostasis expected from reverse-mode $I_{Na/Ca}$ as well as subsequent contractile dysfunction (Janse, 1999).

After application of 5 nM ATXII, diastolic calcium was increased to 125.2 ± 4.03 % of control at the 7 min time point and 119.1 ± 2.45 % of control at the 12 min time point (Figure 12a). When resveratrol (50 μM) was present at the beginning of the experiment, the ATXII-induced elevation in diastolic calcium was prevented (94.9 ± 1.28 % of control) at the 7 min time point. Subsequent removal of resveratrol allowed ATXII to elevate diastolic calcium to 118.5 ± 6.08 % of control at the 12 min time point (Figure 12b). Resveratrol was also found to reverse the effects of ATXII on diastolic calcium: After 5 min treatment with ATXII, diastolic calcium was elevated to 137.7 ± 3.99 % of control. Addition of resveratrol at this time point (7 min) reduced diastolic calcium to 95.9 ± 2.14 % of control at the 12 min time point (Figure 12c).

Myocyte contractility

While large sustained elevations in intracellular calcium eventually lead to myocyte hypercontracture, this is usually preceded by alterations in contractile function that may result from the generation of afterdepolarizations manifested as premature

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contractions. Accordingly, the effects of ATXII and resveratrol on the contractile behavior of field-stimulated myocytes were measured. ATXII (3 nM) induced dysfunction in cardiomyocyte contractility 138.9 ± 7.48 s after control (Figure 13a, c). The addition of 100 μM resveratrol during the first 5 min of ATXII treatment delayed the initiation of contractile dysfunction 3-fold (457.1 ± 26.5 s after the end of control) (Figure 13b, c). Analysis of the frequency of abnormal contractions that exhibited premature peaks indicative of afterdepolarizations revealed that in the presence of ATXII, resveratrol reduced the occurrence of these abnormal contractions from 69.5 ± 10.2 % to 0.71 ± 0.71 % (Figure 13d).

NCX1.1 block

Increases in late I_{Na} lead to elevated intracellular sodium that is thought to favour reverse-mode $I_{Na/Ca}$ and the subsequent influx of calcium into the myocytes (Ravens & Himmel, 1999). Therefore, a plausible alternative explanation for the effects of resveratrol may involve a direct inhibition of reverse-mode $I_{Na/Ca}$. In order to test this experimentally, recombinant rat NCX1.1 was expressed in tsA201 cells and NCX1.1 current was measured in the absence and presence of resveratrol (Figure 14a). Resveratrol, at the concentration (50 μM) shown to reduce calcium loading in myocytes, had no significant effect on reverse-mode NCX1.1 current (Figure 14b).

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Figure 12. Myocyte calcium transients

a–c. Representative recordings of calcium green-1AM fluorescence during a 2 min control period followed by a 10 min treatment period. Expanded traces show calcium transients at the 2 min, 7 min and 12 min time-points. **a.** Calcium transient recording in which 2 min control period is followed by 10 min of 5 nM ATXII alone. **b.** Representative recording of fluorescence showing calcium transients during 2 min control, 5 min of treatment with 5 nM ATXII plus 50 μM resveratrol and 5 min of treatment with 5 nM ATXII. **c.** Representative recording of fluorescence showing calcium transients during 2 min control followed by 5 min of treatment with 5 nM ATXII and 5 min of treatment with 5 nM ATXII plus 50 μM resveratrol. **d.** After the first 5 min of treatment, 5 nM ATXII alone increased diastolic calcium (a), whereas the addition of 50 μM resveratrol during the first 5 min of treatment prevented an increase (b) ($^{**}P <$ 0.001). **e.** At the end of treatment, 5 nM ATXII alone increased diastolic calcium (a); addition of 50 μM resveratrol during the final 5 min of treatment reversed ATXII-induced changes (c) ($^{*}P < 0.05$). n = 6 in all groups. **Experiments performed by Dr. István Baczkó.**

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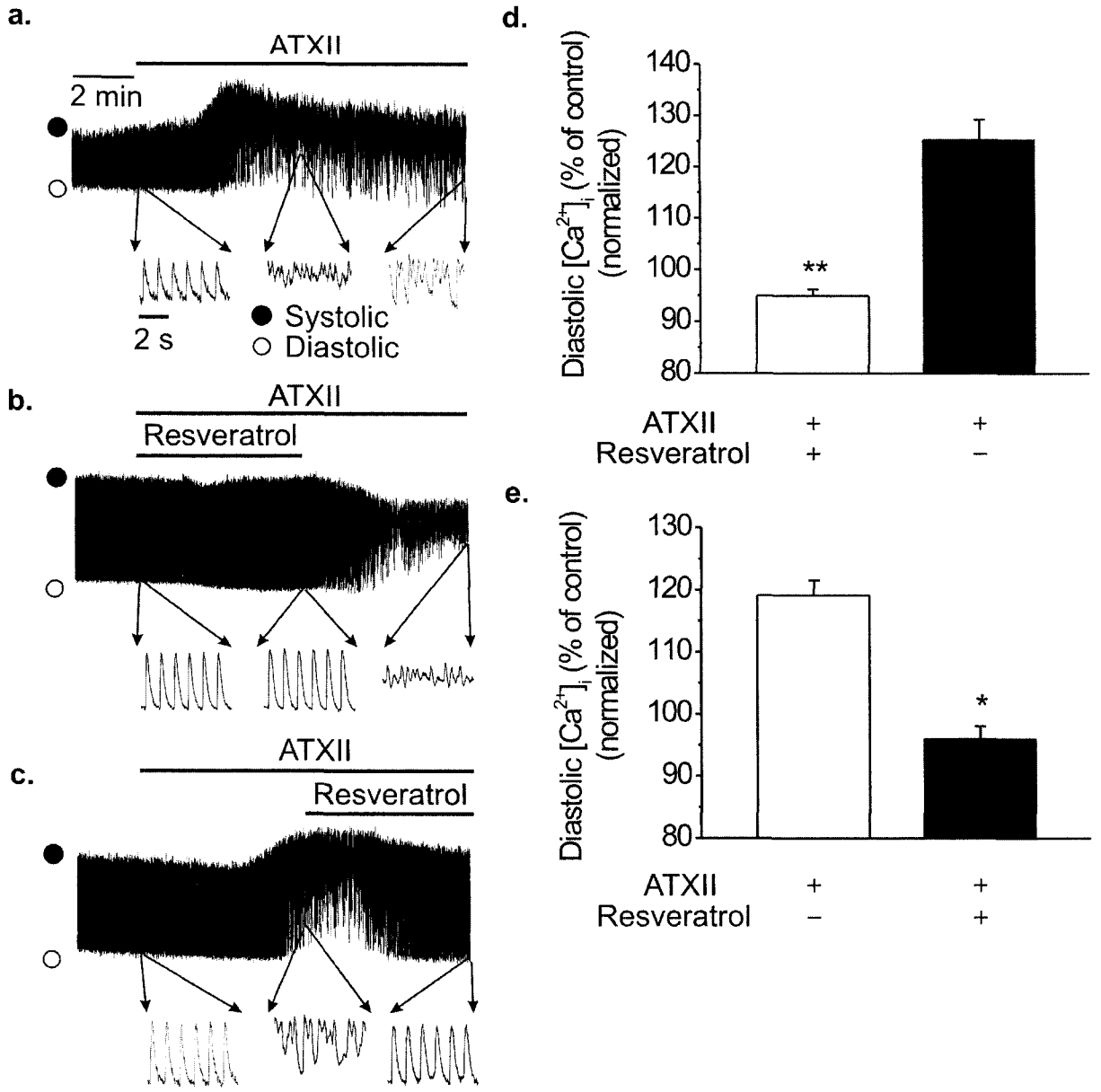

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Figure 13. Myocyte contractility

a-b. Representative recordings of cardiomyocyte shortening. Expanded traces show contractility at the 2 min and 4 min time-points. **a.** 3 nM ATXII produces contractile dysfunction. **b.** Contractile dysfunction occurs later and to a lesser extent in the presence of both 3 nM ATXII and 100 μM resveratrol. **c.** 3 nM ATXII produces contractile dysfunction; with application of 100 μM resveratrol during the first 5 min of ATXII treatment, contractile dysfunction is significantly delayed ($P < 0.001$). **d.** 3 nM ATXII produces contractile dysfunction; with the addition of 100 μM resveratrol, significantly fewer incidents of contractile dysfunction occur ($P < 0.001$). n = 7 in all groups. **Experiments performed by Lynn Jones.**

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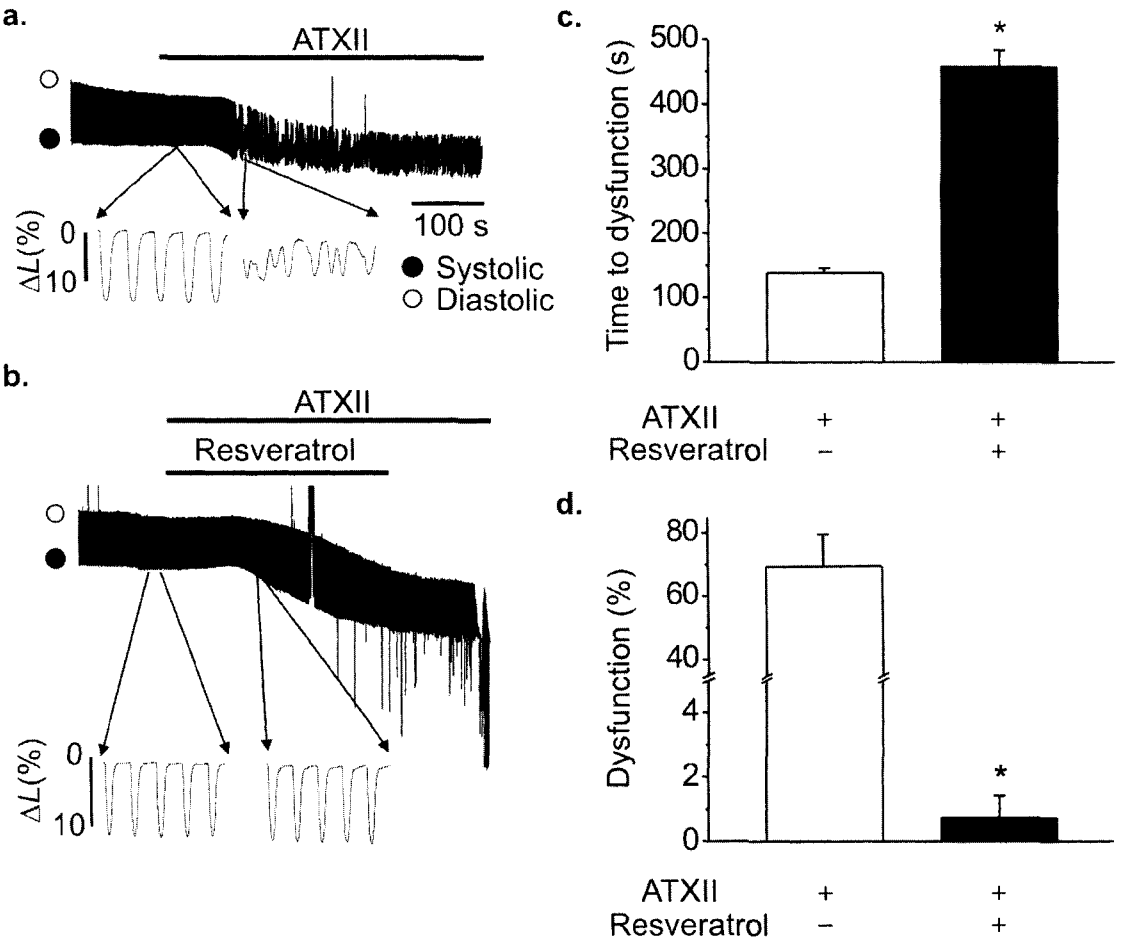

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Figure 14. NCX1.1 block

a. Representative traces of NCX1.1 current in the absence and presence of 50 μM resveratrol. **b.** The ratio of late to peak $I_{Na/Ca}$ in the presence of 50 μM resveratrol (n = 3) is not significantly different from control (n = 3). $P > 0.05$. **Experiments performed by Michael Riedel.**

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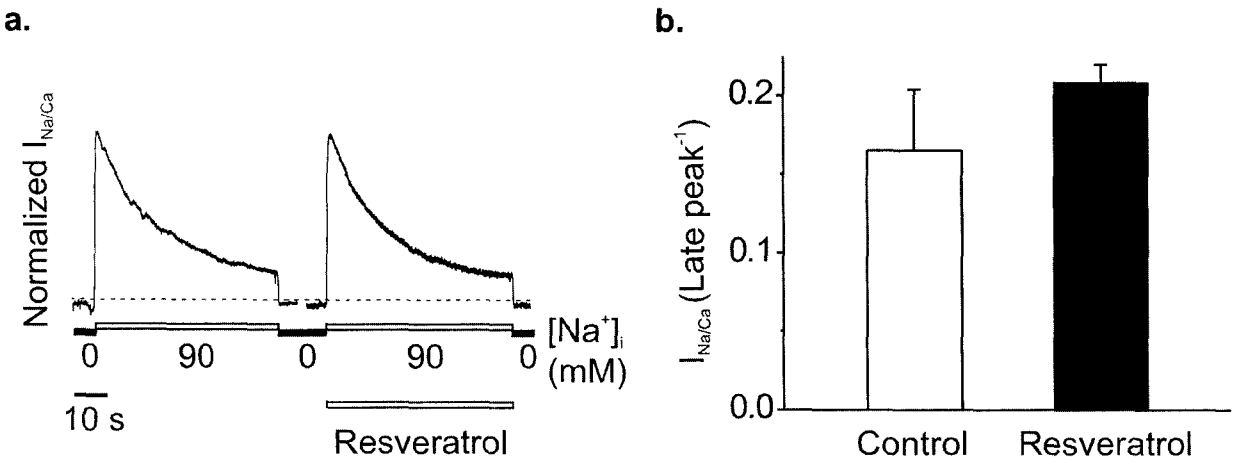

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DISCUSSION

Polyphenol dietary sources and health effects

The foods in human diets, especially those derived directly from plant-based sources, contain numerous polyphenols. These comprise derivatives of benzoic and cinnamic acids as well as lignans, stilbenes and flavonoids, which include flavones, isoflavones, flavanones, flavonols, flavanols and anthocyanidins (Manach *et al.*, 2004). Resveratrol is a stilbene used by fruits as a fungicidal phytoalexin (endogenous pesticide) to protect their exposed skins. Isolation of grape skins reveals a resveratrol content of 5 – 10 % (w w^{-1}) (Gusman *et al.*, 2001). The most common dietary sources of resveratrol are grapes and especially red wine; due to the maceration process used in its manufacture, polyphenols in grape skin are extracted into the wine (Manach *et al.*, 2004). A longer time of maceration leads to increased polyphenol concentration, to a plateau at 12 – 17 d, after which concentration may decrease (Gambuti *et al.*, 2004). Consumption of red wine itself (20 – 30 $g_{alcohol}$ d^{-1}) has been associated with a 40 % reduction in risk of coronary heart disease (CHD) and a diet rich in flavonoids (30 mg d^{-1}) has been linked to a 50 % decrease in CHD mortality (Renaud & de Lorgeril, 1992).

Catechin and quercetin are both flavonoids found in abundance in red wine and in other foods. Quercetin is found in onions at levels of 0.3 – 0.5 g kg^{-1} (Formica & Regelson, 1995), and in berries and green vegetables; Catechins are found in chocolate (0.5 – 0.6 g kg^{-1}), beans, various fruits and tea (Manach *et al.*, 2004) (Table 1).

In addition to the benefits of consuming foods containing these substances, many extracts from inedible plants used for centuries in the folk medicine traditions of

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Australasia, Africa and the Americas have recently been shown to contain active ingredients with polyphenolic activities similar to those described above. For example, resveratrol itself has been isolated from *Yucca schidigera* bark; *Erythophleum lasianthum*, a traditional South African medicinal plant; *Cassia quinquangulata* stems; and the roots of *Polygonum cuspidatum*, a weed used in traditional Chinese and Japanese medicine (known as Hu Zhang and Ko-jo-kon, respectively) for dermatitis, athlete's foot and hyperlipidemia, among many other conditions (Gusman *et al.*, 2001).

Polyphenols are known to provide many of their health benefits via free radical scavenging effects. Resveratrol, for example, inhibits low-density lipoprotein oxidation (Brito *et al.*, 2002; Frankel *et al.*, 1993) and reduces cell injury associated with reperfusion (Hung *et al.*, 2002) through this mechanism. While these effects are attributed to the well-documented antioxidant properties of these polyphenols, it has emerged in recent years that these compounds also interact with intracellular and cell-surface proteins including ion channels. Polyphenols upregulate endothelial nitric oxide synthase (Parks & Booyse, 2002; Wallerath *et al.*, 2002) and endogenous cardiomyocyte antioxidants (Cao & Li, 2004) via possible phytoestrogenic effects and activate or inhibit ionic currents in a multitude of cell types, such as calcium currents in platelets and potassium currents in epithelium (Li *et al*, 2000; Orsini *et al.*, 2004; Dobrydneva *et al*, 2002). Polyphenols prevent the release of reactive oxygen species and inflammatory mediators from polymorphonuclear leukocytes (Rotondo *et al.*, 1998) and protect against atherosclerosis and thrombosis by inhibiting platelet aggregation (Bertelli *et al.*, 1995; Pace-Asciak *et al.*, 1995). The incidence of arrhythmias associated with I/R injury is also

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thought to be due to a non-antioxidant mechanism, possibly involving ion channel modulation (Hung *et al.*, 2004).

Non-antioxidant properties of resveratrol, quercetin, catechins

The presence of several aromatic rings in the structure of polyphenols increases the capacity of these molecules to form resonance structures, thus allowing them to very effectively stabilize scavenged free radicals. The main health benefits of polyphenols, against cancer and cardiovascular disease, have been attributed for the most part to their actions as antioxidants, with other health effects being seen as secondary to these actions. However, in recent years, research has begun to focus more on their direct interactions with cell signaling molecules and ion channels. For instance, in cardiac cell lines, resveratrol has been shown to induce endogenous antioxidants and antioxidative enzymes, possibly via transcriptional mechanisms (Cao & Li, 2004) and in a human endothelial cell line, resveratrol incubation stimulated Ca^{2+}-activated I_K independently of $[Ca^{2+}]_i$; thus potentially via direct ion-channel-modulating effects (Li *et al*, 2000); an effect on small- and large-conductance I_{KCa} was confirmed with antinociceptive tests (Granados-Soto *et al.*, 2002). Ginkgolide components of *Ginkgo biloba* extract inhibit I_{K1} and activate I_{Ca} and I_K while quercetin inhibits I_{Ca} and I_{K1} and activates I_K (Satoh, 2004) leading to and overall prolongation of the action potential. Resveratrol upregulates eNOS mRNA through a phytoestrogenic mechanism (Wallerath *et al*, 2002) and iNOS through inhibition of lipopolysaccharide-mediated NFκB (Tsai *et al.*, 1999) or PKC (Cho *et al.*, 2002). Resveratrol inhibits endothelin-1-dependent and -independent protein tyrosine kinase and mitogen-activated protein kinase phosphorylation (El-Mowafy & White,

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1999) and has shown agonistic activity on the estrogen receptors ERα and ERβ and antagonistic activity at ERα (Bowers *et al.*, 2000). Resveratrol has been shown to inhibit peak I_{Na} in dorsal root ganglion neurons, a potentially analgesic effect (Kim *et al*, 2005) and resveratrol and other phytoestrogens, such as the soybean isoflavones genistein and daidzein, are thought to inhibit calcium influx into platelets via direct interaction with calcium channels; their structural similarity to calcium channel blockers has been explored as an explanation for this interaction (Dobrydneva *et al*, 2002).

Peak and late I_{Na} block by grape extract/polyphenols

In addition to the above effects, this work shows that several dietary polyphenols directly inhibit cardiac VGSCs and that this mechanism may contribute to the cardioprotective effects of these polyphenols documented in such situations as I/R induced arrhythmias (Hung *et al.*, 2004). The application of grape extract at a nutritionally relevant concentration of 15 μg mL^{-1} (Manach *et al.*, 2004) significantly inhibits wild-type VGSCs (Figure 6a, b). Three of the most common polyphenols in grape extract are quercetin, catechin and resveratrol, and these polyphenols, in that order of potency, dose-dependently inhibit peak I_{Na} (Figure 7 e).

While block of peak cardiac I_{Na} may be useful in applications such as cardioplegia (Chambers, 2003) in patients not predisposed to LQT3 (Booker *et al.*, 2003), a balance toward greater inhibition of the late component of I_{Na} found in pathophysiological conditions such as I/R injury and LQT3 would be a more useful approach toward treating these and other complications involving similar ionic imbalances. This work now provides direct evidence that the polyphenol resveratrol may exert some of its

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cardioprotective efficacy via inhibition of late I_{Na} based on the following observations: 1) using the LQT3 R1623Q mutant VGSC as a model, resveratrol inhibits late I_{Na} to a 2-fold greater extent than peak I_{Na} and quercetin showed no difference in its effect on peak and late I_{Na} (Figure 10d); 2) in a parallel model of late I_{Na} using ATXII, resveratrol was found to preferentially inhibit this late I_{Na} compared to peak I_{Na} (Figure 11b) in a manner similar to that observed with the R1623Q mutant, while again, quercetin showed similar inhibition of late and peak I_{Na}.

In addition to their protective effects on blood vessels in preventing atherosclerosis and thrombosis and thereby potentially avoiding ischemia and MI, these results now show that polyphenols may have further actions after the onset of ischemia and/or during reperfusion while still providing antioxidant effects. They may be applied as nutritional agents or as drugs designed to maintain their multiple functions as free radical scavengers, phytoestrogens and voltage-gated channel inhibitors.

Implications in LQT3 and ischemia/reperfusion injury

There is growing interest in the pathophysiological role of late I_{Na} in the heart and selective inhibition of this current has displayed cardioprotective efficacy. The induction of late non-inactivating I_{Na} may lead to prolonged APD, especially in midmyocardial M cells. The difference in the APD in myocardial tissue subtypes (dispersion of repolarization) creates a region that is refractory to further excitation. Increased APD also predisposes to the formation of early afterdepolarizations, which may lead to ectopic beats and/or triggered activity. These, together with the dispersion of repolarization, prime myocardial tissue for the re-entrant loops and spiral waves of excitability

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characteristic of torsade de pointes (Yap & Camm, 2003; Belardinelli et al., 2003). This type of ventricular tachycardia may be fatal, especially if it degrades into ventricular fibrillation, in which the spiral wave breaks up into multiple smaller waves (Sanguinetti & Bennett, 2003). The ability of grape polyphenols, specifically resveratrol, to preferentially inhibit late I_{Na} has direct relevance to disease states such as LQT3, I/R injury and arrhythmias resulting from these. In LQT3, prolongation of APD can be attenuated by reducing late I_{Na}, counteracting the effects of mutations in regions critical to inactivation or activation/inactivation coupling, leading to a lower likelihood of after-depolarization formation and subsequent torsade de pointes arrhythmias (Kambouris *et al.*, 2000; Kambouris *et al.*, 1998). In the case of I/R injury, a reduction in sodium load via late I_{Na} block by polyphenols may reduce or prevent increases in intracellular calcium via reverse-mode $I_{Na/Ca}$, reducing either cellular damage in the form of irreversible cellular hypercontracture via $[Ca^{2+}]_i$-dependent myofilament crossbridge cycling (Quaife *et al.*, 1991) and/or opening of the mPTP on reperfusion (Honda *et al.*, 2005), or arrhythmias generated in a similar manner to the mechanism above. Our results support the notion that block of late I_{Na} may lead to better cellular outcomes downstream, as resveratrol both prevents and reverses ATXII-induced increases in diastolic calcium (Figure 12b–e). Resveratrol also produces a 3-fold delay in ATXII-induced contractile dysfunction (Figure 13c) and dramatically reduces the incidence of abnormal contractions (Figure 13d).

During I/R injury, increases in intracellular sodium via late I_{Na}, in addition to higher NHE activity and lack of Na^+/K^+-ATPase activity are thought to facilitate calcium overload via reverse-mode $I_{Na/Ca}$ (Lazdunski *et al*, 1985; Tani & Neely, 1989; Eigel &

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Hadley, 2001). One further possible mechanism by which protection from this overload might take place is thus via direct inhibition of $I_{Na/Ca}$ by resveratrol. However, the observed lack of effect of on reverse-mode NCX1.1 current (Figure 14) together with our observations on its inhibition of late I_{Na}, suggests that the beneficial effects of resveratrol on dysfunctional calcium handling and contractility induced by ATXII involve a specific inhibition of persistent VGSC, leading indirectly to a reduced reverse-mode $I_{Na/Ca}$. The lack of effect of resveratrol on the cessation of synchronously stimulated calcium transients and contractility in myocytes also indicates that this polyphenol is acting primarily through late and not peak VGSC inhibition.

K_V1.5 block by grape extract/polyphenols: implications in atrial arrhythmias

While K_V1.5 delayed rectifier potassium channels are expressed in both atrial and ventricular tissue types (Fedida *et al.*, 2003), they show more functional expression in atria. The I_{Kur} current, which this channel underlies, is thus a target for atrial specific therapies. The above results show that the red wine polyphenol resveratrol and the green tea polyphenol EGCG both inhibit K_V1.5. Other polyphenols, such as genistein, from soybean, and its derivative, AG-1478; papaverine, from poppies; and oxypeucedanin, a furocoumarin derivative, have also been shown to inhibit these channels (Choi *et al.*, 2002; Choe *et al.*, 2003; Eun *et al.*, 2005), increasing atrial action potential duration and potentially preventing or treating atrial fibrillation. While several inhibitors of atrial potassium current exist for the treatment of atrial fibrillation, including class III antiarrhythmics such as sotalol, amiodarone, ibutilide and dofetilide, even relatively selective class III agents such as dofetilide inhibit more than one type of delayed rectifier

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current (Boriani *et al.*, 2004). Agents to block I_{Kur} exclusively or at significantly lower doses therefore hold more promise in terms of safety and side effect profiles, having less opportunity for proarrhythmia. The atrial selectivity of I_{Kur} blockers appears to be unaffected by additional inhibition of I_{to} and I_{KACh}, however, inhibition of I_{K1}, I_{Kr} or I_{Na} may lead to loss of atrial selectivity and increased risk of ventricular arrhythmias (Brendel & Peukert, 2003). Polyphenolic compounds with dual I_{Na} and I_{Kur} blocking properties thus might not be as effective in this pathophysiological context, especially given the action potential shortening effects of late I_{Na} block. The experimental compounds AVE 0118, S9947 and S20951 are dose-dependently selective for I_{Kur} and seem to be more effective at inhibiting atrial tachyarrhythmias (Knobloch *et al.*, 2004) and the novel antiarrhythmic RSD 1235, which inhibits atrial potassium currents as well as peak I_{Na}, converts 61% of AF with no increase in incidence of proarrhythmia (Boriani *et al.*, 2004). It remains to be determined whether any dietary polyphenols or drugs rationally designed based on these chemical structures could indeed be found to inhibit only atrial voltage-gated potassium channels.

Structure/function relationship in VGSC block by polyphenols

Polyphenols share the common structural feature of a substituted aromatic ring with known VGSC blockers (Figure 5). This feature is thought to be responsible for binding of local anesthetics and antiarrhythmics to their target site, a cluster of hydrophobic residues at the interface of the four S6 segments which line the VGSC pore (Haeseler *et al.*, 2001; Ragsdale *et al.*, 1994; Yarov-Yarovoy *et al.*, 2002). Groups have differed in their assessment of which residues are involved; models propose I409, L965,

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N966, I969, I1760, F1764 and V1774 for etidocaine binding (Yarov-Yarovoy *et al.*, 2002) and I424, I425, G428, I782, V786, L1280, I1284, I1575, F1579 and Y1586 for lidocaine binding (Kondratiev & Tomaselli, 2003). In studies in which lidocaine is chemically "dissected" into its hydrophilic and hydrophobic components (Haeseler *et al.*, 2002; Haeseler & Leuwer, 2002; Zamponi & French, 1993), the polar, amine portion exhibits fast, use-dependent open-state block (Zamponi & French, 1993) while the phenolic structural component of lidocaine, (phenol, aniline, or the analogue 2,6-dimethylphenol), as well as benzocaine, a phenolic local anesthetic with a simpler side chain structure than lidocaine, exhibits no frequency dependence in its inhibition of I_{Na} (Haeseler *et al.*, 2002; Zamponi & French, 1993). Our finding that resveratrol does not incur use-dependent block of VGSCs further confirms the importance of the charged amine moiety in the use-dependent block by lidocaine. It has been theorized that lack of use-dependence of resveratrol could be related to its rapid dissociation from the VGSC (Kim *et al.*, 2005), however, our observations on the lack of washout of resveratrol from VGSCs indicate this to be unlikely. Furthermore, the above data indicate that, as with simple phenolics, the voltage-dependence of conductance and steady-state availability of VGSCs are not affected by polyphenols (Figure 8).

A key finding from this work is the ~2-fold selectivity of resveratrol for late over peak I_{Na} compared with the absence of selectivity in the case of quercetin. An analysis of their structures (Figure 5a, c) suggests that quercetin's bulkier structure, richer in electron-donating groups, may sterically hinder interaction with residues deeper in the VGSC's pore responsible for inactivation state stabilization (Ragsdale *et al.*, 1994;

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Yarov-Yarovoy *et al.*, 2002) and/or for allowing necessary conformational changes for a late current to result (Leuwer *et al.*, 2004; Yarov-Yarovoy *et al.*, 2002).

In addition, the observation that quercetin is a much more effective VGSC inhibitor than catechin (IC_{50} values of 19.4 μM and 76.8 μM respectively) despite almost identical structures suggests that the presence of the conjugated carbonyl on quercetin's second ring structure may contribute to the observed increase in VGSC inhibitory efficacy, perhaps via more favourable hydrogen bonding to amino acids proximal to those in the local anesthetic/antiarrhythmic target site. Due to its preferential inhibition of persistent current resveratrol was used in further studies involving late sodium current.

A 20-fold increase in late current was seen with the application of ATXII to wild-type sodium channels and resveratrol was found to inhibit this persistent sodium current 3-fold more than peak wild-type current (Figure 11). ATXII was used again in subsequent studies to pharmacologically simulate situations of sodium overload in isolated rat right ventricular myocytes so as to examine the protective effects of resveratrol exclusively in terms of its sodium channel-blocking properties. It was demonstrated that resveratrol prevents and reverses ATXII-induced increases in diastolic calcium (Figure 12) and delays ATXII-induced contractile dysfunction by 3-fold (Figure 13). The specificity of ATXII for the sodium channel implies that sodium channel block is at least partially responsible for the effects of resveratrol on calcium homeostasis. To determine whether there could be any additional contribution of inhibition of reverse-mode sodium-calcium exchanger activity, the effect of resveratrol on current through NCX1.1 was measured. Exchanger current was found to be unaffected by the presence of 50 μM of the polyphenol (Figure 14) indicating that additional protection via this

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mechanism was unlikely. Limiting sodium overload alone may attenuate a rise in intracellular calcium by reducing the driving force for reverse-mode sodium-calcium exchange.

Additional benefits of resveratrol and other red grape polyphenols are also likely contributed by the well-documented antioxidant properties of the polyphenols in reducing reperfusion-induced free-radical damage (Hung *et al.*, 2002; Valdez *et al.*, 2002). During I/R injury, high levels of production of radicals relative to their rate of elimination by scavenging or enzymatic modification create oxidative stress. Lipids, proteins and nucleic acids are peroxidated, damaging ion channels and membranes. Polyphenols scavenge peroxyradicals (Alvarez *et al.*, 2004; Valdez *et al.*, 2002) and this may contribute to alterations in ion currents seen by application of antioxidants in a setting of I/R or hypoxia/reoxygenation (Eigel *et al.*, 2004). Changes in redox potential or surface charge may also account for some ionic current block (Bhatnagar *et al.*, 1990), thus it is possible that the antioxidant properties of polyphenols may contribute to the observed VGSC inhibition. However, parallel experiments with N-acetylcysteine, a structurally unrelated antioxidant lacking a substituted phenolic group (Figure 5f), demonstrated no effect on I_{Na} (Figure 7e), indicating that this may not be a significant mechanism in this case.

Dietary/clinical implications

Polyphenol concentrations effective for free radical scavenging are in the 5–20 μM range *in vitro* (Alvarez *et al.*, 2002; Leonard *et al.*, 2003) and in perfused rat hearts showing ischemic recovery (Ray *et al.*, 1999), indicating potentially clinically relevant doses. While concentrations of individual polyphenols in the 50 μM range, as used in these experiments, are unlikely to be reached in human plasma after moderate red wine

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consumption (Vitaglione *et al.*, 2005), a polyphenol-rich diet may include several dietary sources, raising the total polyphenol concentration above that for each compound alone, likely imparting additive effects on inhibition of I_{Kur} and I_{Na}. The relatively low concentration of grape extract—a source of several polyphenols—necessary for an inhibitory effect is evidence of this.

The observations that grape extract and resveratrol are resistant to washout (Figures 6b and 9d) indicate that membrane partitioning and subsequent binding to VGSCs may contribute to the efficacy of these polyphenols even at lower apparent plasma concentrations. This is supported by evidence suggesting that dietary polyphenols may accumulate in tissues, resulting in a higher local concentration of these compounds. For example, resveratrol concentrations are 2.4-fold higher in mouse liver and heart than the concentrations reached in plasma (Sale *et al.*, 2004).

Moreover, many polyphenols exist in both aglycone and glycoside forms in plasma (Manach *et al.*, 2004; Vitrac *et al.*, 2003) and the latter are cleaved by endogenous glucosidase activity to increase polyphenol bioavailability. Quercetin glucosides, abundant in onions, are very well absorbed in the intestine via two mechanisms. Firstly, they may be transported into enterocytes and cleaved there by β-glucosidase; secondly, they may be cleaved by a lactase hydrolase and then diffuse into the cell. Additionally, the slow rate of elimination of the flavonols indicates that an elevated plasma level could be reached with sustained intake. EGCG is the only catechin to remain unaltered in plasma; others such as epigallocatechin and epicatechin are sulfated and/or glucuronidated, as well as being modified by microbial valerolactone moieties. These have antioxidant properties and long half-lives, potentially enhancing and

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prolonging the effects of some polyphenols (Manach *et al.*, 2005). Resveratrol has shown low plasma levels in several studies, but is also modified by sulfation and glucuronidation, therefore active concentration may be greater than previously suggested (Wenzel & Somoza, 2005). Furthermore, there is evidence that quercetin and other flavonoids could inhibit the glucuronidation of resveratrol (de Santi *et al.*, 2000). Regular red wine consumption provides resveratrol concentrations in the 25 – 30 μM range in mouse liver (Vitrac *et al.*, 2003), and while concentrations in the heart in the cited study were moderate, mouse liver and heart activities were comparable in another study, where they were 2.4-fold greater than concentrations reached in plasma (Sale *et al.*, 2004). It is also possible that *in vivo*, effects of polyphenols on other ion channels such as potassium and calcium channels may further contribute to cardioprotection, and that protective mechanisms acting at other sites in the cardiovascular system may have important impacts on contributions of dietary polyphenols to overall health.

Proposed mechanism: late I_{Na} inhibition

These results demonstrate that several common dietary polyphenols are effective inhibitors of peak and/or late I_{Na} and improve myocyte calcium handling and contractility downstream of inhibition of late I_{Na}, suggesting that this may account for their effectiveness at reducing arrhythmias as well as damage caused by I/R injury (Hung *et al.*, 2004). The major ionic changes seen during I/R injury are illustrated in Figure 4. $[K^+]_o$ is increased via excess K^+ efflux, decreased washout and Na^+/K^+-ATPase inhibition; this, as well as NHE activity due to lower pH_i and VGSC modification to produce a late I_{Na} cause an elevated $[Na^+]_i$; reverse mode NCX leads to a potentially

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damaging increase in and overload of $[Ca^{2+}]_i$, potentially leading to torsade de pointes and/or cell death. Reactive oxygen and nitrogen species also play an important role in the cellular damage caused by reperfusion injury and in the generation of arrhythmias. These processes may be attenuated by dietary polyphenols, not only due to their free radical scavenging properties, but as shown by this work, via inhibition of late I_{Na}, protecting the heart against subsequent $[Ca^{2+}]_i$ increases leading to hypercontracture (Figure 15). The fact that polyphenols such as resveratrol form an important part of the makeup of red wine indicates that this mechanism may contribute to the observed protective of effects of grape/red wine ingestion on cardiac function during I/R injury.

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Figure 15. Proposed mechanism

a. Schema of ionic currents in an ischemic myocyte and proposed mechanism of action of dietary polyphenols. (1): During ischemia, the [ATP]/[ADP][P_i] ratio is decreased and glycolysis is increased. (2): While sodium-potassium pump inhibition and sodium-hydrogen exchange may still occur, inhibition of persistent (late) sodium current by polyphenols (3) reduces sodium overload; there is thus less driving force for calcium entry via (4) reverse-mode sodium-calcium exchange, (5) diminishing calcium overload and (6) reducing myocardial damage.

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a.

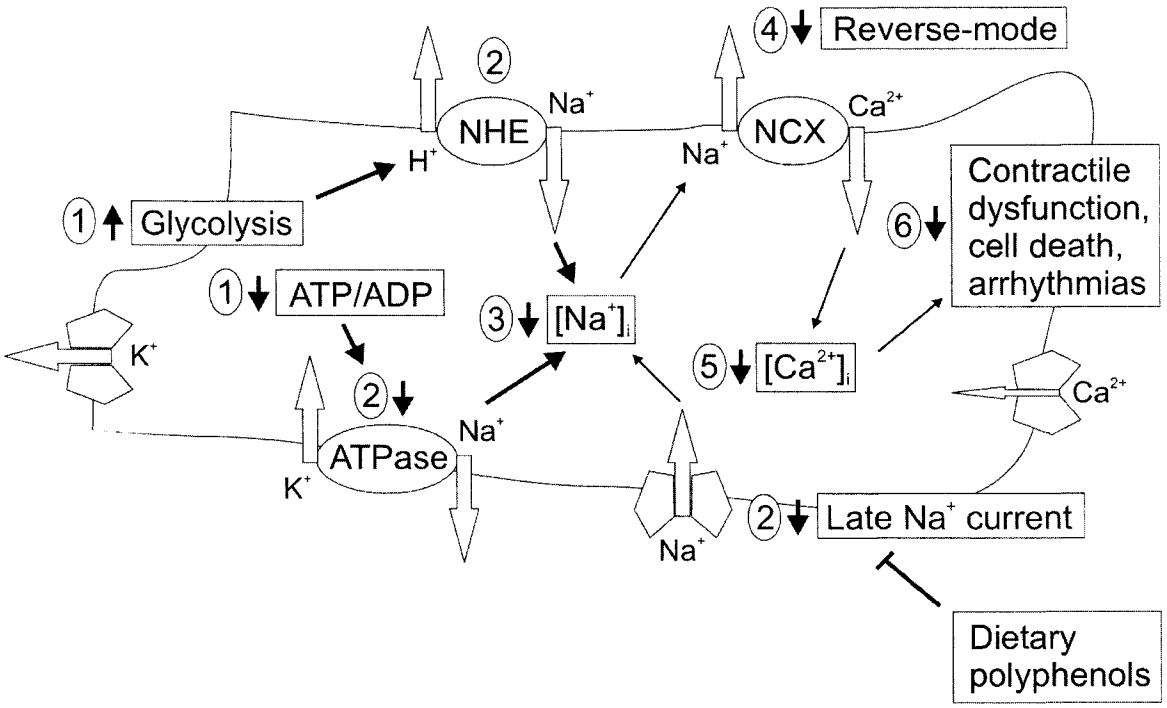

Figure 15.

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FUTURE DIRECTIONS

Given the important role that ion channels such as $Na_V1.5$ and $K_V1.5$ play in the regulation of intra- and intercellular communication, and the role that their dysfunction plays in I/R injury and LQT3 and in atrial arrhythmias, respectively, in addition to a variety of other disease states, further studies on the modulatory effects of dietary polyphenols on these and other types of ion channels are definitely warranted.

These studies have shown the basis for novel mechanisms of cardioprotection by polyphenols in addition to some of those already described in the literature. Unfortunately, these mechanisms were only characterized to the extent that time allowed and work remains to be done to elucidate these pathways. Further studies in myocytes, to examine the effects of polyphenols on native currents and on action potential parameters, will be performed. The expression of recombinant $Na_V1.5$ only employed the α subunit, therefore further studies will use coexpression of one or more β subunits. Other biophysical parameters such as steady-state and time constants of activation and inactivation would help to further describe sodium and potassium channel block. Frequency-dependence studies were preliminary, and further improvements in study design might shed light on unanswered questions regarding the time- and frequency-dependence of washout and the differences between peak and late current block.

In reperfusion injury, new treatments are sought that provide benefits against the cellular damage caused by oxidative stress in addition to those that prevent life threatening arrhythmias. The free-radical scavenging properties of polyphenolic compounds, together with these newly described effects on late sodium current, provide a

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possible basis for the development of bifunctional compounds to treat the widespread phenomenon of ischemia-reperfusion injury.

In LQTS, there is potential for the application of mutation-specific therapies using polyphenolic compounds or their rationally designed derivatives; for this, further study of the actions of polyphenols in cells expressing mutant sodium and potassium channels will be required. For example, the R1193Q SCN5A mutation, differentially expressed between racially distinct groups, raises the question of whether inherent protection could be provided by cultural (i.e. dietary) as well as genetic similarities within populations. Experiments performed as part of these studies involving a model of LQT3 late sodium current unfortunately only examined one mutant model, in which the mechanism of late current generation (uncoupling of activation and inactivation), is less common than some other forms of LQT3. Further work should characterize late sodium current block by polyphenols using other well-described LQT3 mutations.

In atrial fibrillation, a major focus of new drug development is on testing atrial-selective potassium channel blockers. Future research in this area could involve determining the I_{Kur}-specificity of potassium channel block by certain polyphenols or polyphenolic derivatives. Our work examined only inhibition by two polyphenols at a preliminary level. Further studies should test a variety of polyphenols at varying concentrations.

In conclusion, this research opens up new pathways of investigation into the cardiovascular benefits of polyphenolic compounds and their dual actions as antioxidants and ion channel modulators.

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