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RESEARCH PAPER

Inhibition of cardiac voltage-gated sodium channels by grape polyphenols

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Background and purpose: The cardiovascular benefits of red wine consumption are often attributed to the antioxidant effects of its polyphenolic constituents, including quercetin, catechin and resveratrol. Inhibition of cardiac voltage-gated sodium channels (VGSCs) is antiarrhythmic and cardioprotective. As polyphenols may also modulate ion channels, and possess structural similarities to several antiarrhythmic VGSC inhibitors, we hypothesised that VGSC inhibition may contribute to cardioprotection by these polyphenols.
Experimental approach: The whole-cell voltage-clamp technique was used to record peak and late VGSC currents (I_{Na}) from recombinant human heart $Na_V1.5$ channels expressed in tsA201 cells. Right ventricular myocytes from rat heart were isolated and single myocytes were field-stimulated. Either calcium transients or contractility were measured using the calcium-sensitive dye Calcium-Green 1AM or video edge detection, respectively.
Key results: The red grape polyphenols quercetin, catechin and resveratrol blocked peak I_{Na} with IC_{50}s of 19.4 μM, 76.8 μM and 77.3 μM, respectively. In contrast to lidocaine, resveratrol did not exhibit any frequency-dependence of peak I_{Na} block. Late I_{Na} induced by the VGSC long QT mutant R1623Q was reduced by resveratrol and quercetin. Resveratrol and quercetin also blocked late I_{Na} induced by the toxin, ATX II, with IC_{50}s of 26.1 μM and 24.9 μM, respectively. In field-stimulated myocytes, ATXII-induced increases in diastolic calcium were prevented and reversed by resveratrol. ATXII-induced contractile dysfunction was delayed and reduced by resveratrol.
Conclusions and implications: Our results indicate that several red grape polyphenols inhibit cardiac VGSCs and that this effect may contribute to the documented cardioprotective efficacy of red grape products.
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Keywords: voltage-gated sodium channels; polyphenols; resveratrol; quercetin; catechin; ischaemia-reperfusion injury; sodium homeostasis; calcium homeostasis; calcium overload; cardioprotection

Abbreviations: APD, action potential duration; ATXII, *Anemonia sulcata* toxin II; CHD, coronary heart disease; I_{Na}, voltage-gated sodium channel current; $I_{Na/Ca}$, sodium–calcium exchanger current; I/R, ischaemia-reperfusion; LQT3, long QT syndrome 3; $Na_V1.5$, human heart voltage-gated sodium channel clone; NCX1.1, rat sodium–calcium exchanger clone; VGSC, voltage-gated sodium channel.

Introduction

The rapid generation of net inward sodium current (peak I_{Na}) via voltage-gated sodium channel (VGSC) activation is responsible for the fast initiation of the cardiac action potential. VGSCs normally inactivate within 10 ms and thus sodium entry is usually limited to the depolarization phase of the action potential. However, under several circumstances, the inactivation process is dysfunctional, resulting in the development of a persistent current (late I_{Na}). This is a key feature of several pathologies, including long QT syndrome 3 (LQT3), in which congenital mutations in the SCN5A gene lead to impaired inactivation of cardiac VGSCs, resulting in a proarrhythmic prolonged action potential (Wang *et al*., 1995; Janse, 1999). The increased influx of sodium during the action potential plateau phase may promote the formation of after-depolarizations, leading to severe types of ventricular tachycardia such as torsade de pointes (Janse, 1999). Furthermore, late I_{Na} has been implicated in heart failure and during ischaemia-reperfusion (I/R) injury (Maltsev *et al*., 1998; Xiao and Allen, 1999). The accumulation of cytosolic sodium through persistent VGSC activity may also lead to calcium overload via increased reverse-mode sodium–calcium exchanger current ($I_{Na/Ca}$) (Ravens and Himmel, 1999). This may cause the formation of premature after-depolarizations and triggered activity,

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leading to ventricular arrhythmias as well as irreversible cell injury via calcium loading and subsequent hypercontracture (Janse, 1999).

VGSC inhibitors represent an important class of anti-arrhythmic agents that include lidocaine and mexiletine (Liu *et al*., 2003). Although some of these compounds possess desirable features, such as frequency-dependence of block, they may inhibit peak I_{Na} to the same extent as late I_{Na}. Identification of novel VGSC inhibitors that selectively inhibit late I_{Na} may provide useful, pathology-specific, pharmacological tools. For example, the antianginal compound ranolazine is proposed to act via inhibition of late I_{Na} and provide cardioprotection by a subsequent reduction of calcium overload without altering normal conduction (Antzelevitch *et al*., 2004).

Many bioactive compounds contain one or more phenol rings in their structure and several of them have demonstrated cardioprotective efficacy. In particular, red grape products such as red wine are important sources of several dietary polyphenols with concentrations estimated to range from 0.5 to 200 $\mu g\,ml^{-1}$ for catechin and epicatechin, 0.25–50 $\mu g\,ml^{-1}$ for quercetin and 0.05–8.5 $\mu g\,ml^{-1}$ for resveratrol (Bertelli *et al*., 1998; Ray *et al*., 1999; Leonard *et al*., 2003; Gambuti *et al*., 2004; Manach *et al*., 2004). These polyphenolic constituents are thought to be responsible for the well-documented cardiovascular benefits of red wine and for the 'French Paradox' of low mortality from cardiovascular disease (Renaud and de Lorgeril, 1992).

Resveratrol is a well-characterized grape polyphenol that has exhibited a multitude of cardioprotective properties *in vitro* and *in vivo* (Frankel *et al*., 1993; Bertelli *et al*., 1995; Pace-Asciak *et al*., 1995; Rotondo *et al*., 1998; Ray *et al*., 1999; Hung *et al*., 2000; Cao and Li, 2004), including antiplatelet (Bertelli *et al*., 1995; Pace-Asciak *et al*., 1995), antioxidative (Frankel *et al*., 1993; Cao and Li, 2004), anti-ischaemic (Ray *et al*., 1999; Hung *et al*., 2000) and antiarrhythmic (Hung *et al*., 2000) effects. In models of I/R injury, resveratrol improves functional recovery (Ray *et al*., 1999) and reduces both infarct size (Ray *et al*., 1999; Hung *et al*., 2000) 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 other antioxidant effects of resveratrol but a separate, as yet uncharacterized mechanism may be involved, related to a reduction of I/R-induced arrhythmias (Hung *et al*., 2004), suggestive of an effect on cardiac ion channel function. For example, resveratrol has recently shown inhibition of L-type calcium channels and reductions in action potential duration (Liew *et al*, 2005).

The red grape polyphenols catechin, quercetin and resveratrol share the common structural feature of one or more phenolic rings with VGSC-blocking drugs such as lidocaine (Figure 1c). As the phenol group found in lidocaine is thought to impart late VGSC block (Zamponi and French, 1993; Haeseler *et al*., 2002), we hypothesized that red grape polyphenolic compounds may also inhibit peak and/or late I_{Na}, contributing to the documented beneficial effects of these grape polyphenols. Supporting this hypothesis, it has recently been shown that resveratrol inhibits neuronal VGSCs (Kim *et al*., 2005).

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% foetal 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 50–70% confluence onto 35 mm culture dishes 3–4 h before transfection. Mammalian expression vectors encoding the human heart VGSC clone ($Na_V1.5$) – either wild-type or with the R1623Q mutation (see below) – and green fluorescent protein (pGL, Life Technologies, Burlington, Canada), as a visual marker, were co-transfected into cells using the calcium phosphate precipitation technique. $Na_V1.5$ was generously provided by Dr AM Brown (Case Western Reserve University, Cleveland, OH, USA). Cells were plated at 10–30% confluence onto cover slips 40–45 h after transfection. Single cells were used for electrophysiological recording during 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, Alberta, Canada) and Dr JY Cheung (Geisinger Medical Center, Danville, PA, USA).

$Na_V1.5$ Mutagenesis

Amino acid substitution of an arginine residue with a glutamine at position 1623 (R1623Q) of the $Na_V1.5$ α-subunit was performed using polymerase chain reaction (PCR). A 569 bp cDNA of $Na_V1.5$ was amplified using the oligonucleotide primers '1623SeqF' (5′-AGAGCAGCCTCAG TGGGA-3′) (base pair 4306 at start) and '1623L' (5′-GGCGG ATGACTTGGAAGA-3′) (Operon Biotechnologies Inc., Germantown, MD, USA). A 468 bp cDNA of $Na_V1.5$ was amplified concurrently using the primers '1623U' (5′-GACGCTCT TCCAAGTCAT-3′) and '1623SeqR' (base pair 5330 at end) (5′-ACGCTGAAGTTCTCCAGGA-3′). The two PCR products were purified via gel-extraction and combined in a second round of PCR with the primer pair '1623SeqF' and '1623SeqR'. The resulting 1024 bp PCR product was digested with *Bsr*GI to yield a 960 bp insert, which was then subcloned back into wild-type $Na_V1.5$ to produce the R1623Q construct as confirmed by sequencing.

Electrophysiology

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

1–4 MΩ. The pipette solution contained (in mM): 130 CsCl, 5 NaCl, 5 tetraethylammonium (TEA)-Cl, 2.5 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES), and 1 ethylene glycol-bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (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 were applied to cells using a multi-input perfusion pipette (switch time <2 s). Whole-cell voltage-clamp was used to record macroscopic I_{Na}. Data were recorded using an Axopatch 200B patch-clamp amplifier and Clampex 8 software (Axon Instruments, Foster City, CA, USA). 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. Parallel current–voltage curves were also recorded and data fitted to the Boltzmann equation to yield G_{max} curves. Owing to space-clamp concerns, data were discarded if the resulting slope factor was <6 mV. $I_{Na/Ca}$ measurements were obtained using the inside-out excised-patch patch-clamp technique. Outward reverse-mode currents were elicited by rapidly (<2 s) switching the solution flowing from a multi-input perfusion pipette 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, 5 HEPES, 10 glucose, 2 MgATP, 1.4 $MgCl_2$, 4.28 $CaCl_2$, and 5 EGTA. The pH of these solutions was adjusted to 7.2 with CsOH.

Myocyte isolation

Rats were killed 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 myocytes were obtained from the right ventricle by enzymic dissociation using standard protocols previously described (Shimoni *et al*., 1998). After 1 h, cells were placed on coverslips for observation at $\times 200$ and were 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, USA) for 30 min at room temperature followed by 30 min at 37°C. After loading, cells were washed and placed on coverslips for observation at $\times 200$ with a CK40 inverted microscope (Olympus, Melville, NY, USA). Cells were then superfused with control solution as described above and field-stimulated at 1 Hz. A Photon Technology International Photomultiplier Detection System (PTI, Lawrenceville, NJ, USA) with Clampex 8 software was used for data acquisition. Calcium Green-1AM was excited with 480 nm light, and the emitted light intensity at 520 nm was digitized. Diastolic calcium was measured as a percentage of control and normalized to the peak amplitude of the calcium transient (Baczko *et al*., 2005).

Measurement of cell shortening

Cell shortening was measured using a video edge detector (Crescent Electronics, Salt Lake City, UT, USA). Myocytes were field-stimulated at 1 Hz with 2 ms square pulses at a constant current 20% above threshold value. Cell shortening was 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). Irregularly shaped contractions were defined as dysfunctional and the number of dysfunctional contractions was then assessed as a percentage of total contractile events.

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

Statistics

Data were analysed using Clampex 8, Microsoft Excel and Origin Graph. Data are presented as means $\pm$ s.e.m. or as a fit to the Boltzmann equation. Statistical analyses of data were performed using the Student's paired or unpaired *t*-test or ANOVA as appropriate. $P<0.05$ was considered statistically significant.

Drugs and chemicals

Grape extract (BioVin, Cyvex Nutrition Inc., CA, USA) 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, USA) and were also prepared as $1000\times$ stock solutions in dimethyl sulphoxide: Resveratrol at 10, 20, 50, 100 and 200 mM; quercetin at 1, 2, 5, 10, 20 and 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. Dimethyl sulphoxide (0.1% v v^{-1}) was used in control solutions.

Results

VGSC block

In order to first assess the VGSC blocking properties of grape-derived polyphenols, we tested the effects of grape extract on peak I_{Na} in a recombinant system using tsA201 cells. Application of 15 μg ml^{-1} grape extract showed significant inhibition of peak I_{Na} (Figure 1a and b). As red grape extract and red wine contain a variety of bioactive polyphenolic compounds, we tested the effects of three of the most commonly occurring polyphenols, catechin, quercetin and resveratrol (Figure 1c), individually on recombinant I_{Na} (Figure 2a–c). Concentration-dependence studies (Figure 2e) yielded IC_{50}s for the effects of resveratrol and quercetin on I_{Na} of 77.3 ± 8.20 μM and 19.4 ± 2.05 μM. Catechin inhibited peak I_{Na} with an IC_{50} of 76.8 ± 5.15 μM. Voltage-dependence of activation (G_{max50}) in the presence of 50 μM resveratrol

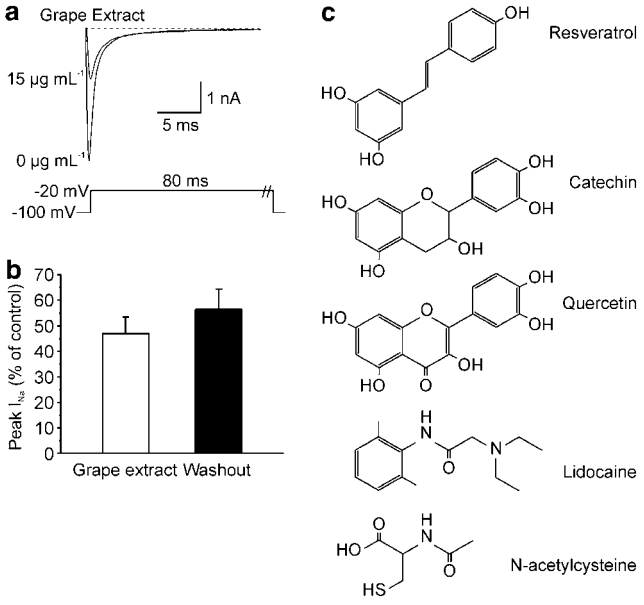

Figure 1 (**a**) Representative traces of recombinant I_{Na} through VGSCs expressed in tsA201 cells in the presence of 0 or 15 μg ml^{-1} grape extract. (**b**) Grape extract (15 μg ml^{-1}, $n=4$, $P<0.05$) inhibited peak recombinant I_{Na} but did not wash out. (**c**) Structural formulae of resveratrol, catechin and quercetin, antioxidant polyphenols with substituted aromatic rings. Lidocaine is a VGSC blocker containing a similar aromatic group. *N*-acetylcysteine is an antioxidant without structural similarity to these compounds.

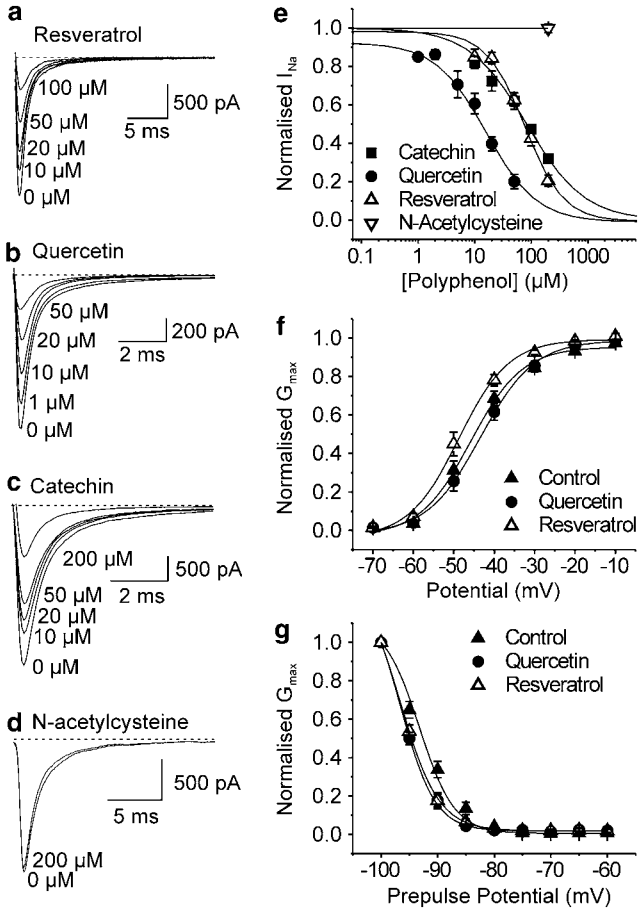

Figure 2 (**a**) Representative recombinant I_{Na} traces in the presence of 0, 10, 20, 50 or 100 μM resveratrol. (**b**) Representative recombinant I_{Na} traces in the presence of 0, 1, 10, 20 or 50 μM quercetin. (**c**) Representative recombinant I_{Na} traces in the presence of 0, 10, 20, 50, or 200 μM catechin. (**d**) Representative traces of recombinant I_{Na} in the presence of 0 or 200 μM *N*-acetylcysteine. (**e**) Concentration–response curves for the block of peak recombinant I_{Na} by catechin, quercetin, resveratrol and *N*-acetylcysteine ($n=4$–11). (**f**) Voltage-dependence of activation at varying test potentials is unchanged with 50 μM resveratrol or 10 μM quercetin present ($n=9$–13). (**g**) Inactivation at varying prepulse potentials is unchanged with 50 μM resveratrol or 10 μM quercetin present ($n=5$).

(-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 2f); nor was steady-state availability, for which G_{max50} was -93.2 ± 0.596 mV for control, -96.6 ± 0.936 mV for quercetin and -96.7 ± 1.21 mV for resveratrol (Figure 2g). To ascertain whether the VGSC blocking effects of these polyphenols could be attributed to the documented antioxidant properties of these compounds, we tested the effects of the structurally unrelated antioxidant *N*-acetylcysteine (Figure 1c, 200 μM) and found that it had no significant effect on I_{Na} (Figure 2d and e).

Frequency-dependence

One of the key properties of the antiarrhythmic agent lidocaine (Figure 1c) is the use- or frequency-dependence of its VGSC block. Therefore, we compared the frequency-dependence of resveratrol's I_{Na} block to that of lidocaine. At the higher stimulation frequency of 10 Hz, resveratrol (50 μM) and lidocaine (50 μM) inhibited peak I_{Na} by 38 ± 7 and 67 ± 4%, respectively (Figures 3a–c). In contrast, at a lower pulse frequency of 0.2 Hz, resveratrol (50 μM) and lidocaine (50 μM) inhibited peak I_{Na} equally (Figure 3a–c). Upon washout, the effect of lidocaine was fully reversible while that of resveratrol was only partly reversible (Figure 3d).

Late I_{Na} *inhibition*

Induction of late noninactivating I_{Na} has been associated with many of the pathological characteristics of I/R injury (Undrovinas *et al*., 1992; Wu and Corr, 1994; Ju *et al*., 1996; Ward and Giles, 1997) and has been suggested to contribute to observed ionic disturbances and consequent electrical and contractile dysfunction (Van Emous *et al*., 1997; Karmazyn *et al*., 1999). In addition, the expression of mutated VGSCs in congenital LQT3 may cause proarrhythmic increases in action potential duration (APD) by increasing noninactivating late I_{Na} leading to an increase in the QT interval and the precipitation of torsade de pointes (Wang *et al*., 1997; Janse, 1999). As inhibition of late VGSC activity has the potential to be cardioprotective and antiarrhythmic, we tested the effects of resveratrol and quercetin on mutant $Na_V1.5$ channels containing the LQT3 mutation R1623Q (Figure 4a). Resveratrol (50 μM) reduced both peak and late I_{Na} but exhibited a higher efficacy for late I_{Na} block in the R1623Q LQT3 $Na_V1.5$ mutant (Figures 4b and d). In contrast, quercetin (10 μM) showed no selectivity between peak and late R1623Q I_{Na} (Figures 4c and d).

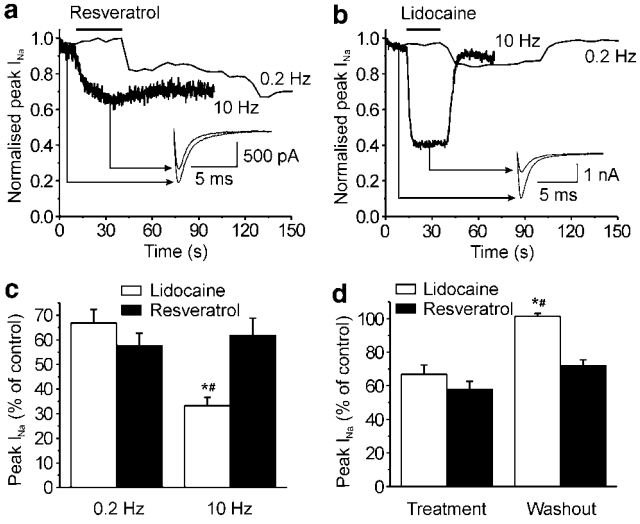

Figure 3 (**a**) Representative time course of peak recombinant I_{Na} before, during and after application of 50 μM resveratrol at a pulse frequency of 10 Hz or 0.2 Hz. (Inset) Representative recombinant I_{Na} traces in the presence of 0 or 50 μM resveratrol at a pulse frequency of 10 Hz. (**b**) Representative time course of peak recombinant I_{Na} before, during and after application of 50 μM lidocaine at a pulse frequency of 10 Hz or 0.2 Hz. (Inset) Representative recombinant I_{Na} traces in the presence of 0 or 50 μM lidocaine at a pulse frequency of 10 Hz. (**c**) At a pulse frequency of 0.2 Hz, resveratrol and lidocaine, at 50 μM each, inhibit peak I_{Na} to a similar extent ($n=6$–7). At a pulse frequency of 10 Hz, lidocaine (50 μM, $n=7$) inhibits peak I_{Na} to a greater extent than does resveratrol (50 μM, $n=7$). (**d**) Peak I_{Na} blocked by 50 μM lidocaine returns to control levels upon washout; peak I_{Na} block by 50 μM resveratrol shows no significant washout ($n=6$). $^{\#}P<0.05$ within groups; $^{*}P<0.05$ between groups.

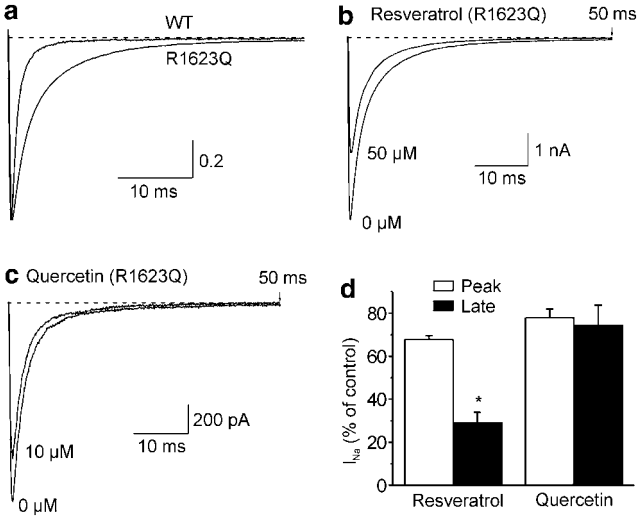

Figure 4 (**a**) Normalized traces comparing I_{Na} from mutant and wild-type VGSCs expressed in tsA201 cells. (**b**) Representative traces of I_{Na} through mutant (R1623Q) VGSCs in the presence of 0 or 50 μM resveratrol. (**c**) Representative traces of I_{Na} through R1623Q VGSCs in the presence of 0 or 10 μM quercetin. (**d**) Peak and late I_{Na} through R1623Q VGSCs in the presence of 50 μM resveratrol or 10 μM quercetin. All groups were normalized to their controls. Late R1623Q I_{Na} was blocked to a greater extent than peak I_{Na} during application of 50 μM resveratrol ($n=7$) and late R1623Q I_{Na} was blocked to the same extent as peak I_{Na} during application of 10 μM quercetin ($n=10$). $^{*}P<0.001$.

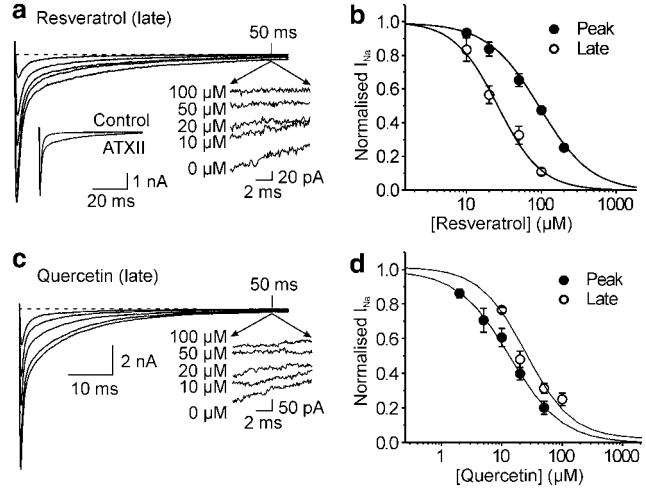

Figure 5 (**a**) Representative recombinant I_{Na} traces in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM resveratrol. (Left inset). Representative recombinant I_{Na} traces in the presence of 0 or 5 nM ATXII. (Right inset) Expanded trace of late I_{Na} in the presence of 5 nM ATXII and 0, 10, 20, 50 or 100 μM resveratrol (40–60 ms after the depolarizing pulse). (**b**) Concentration–response curves for the block of peak I_{Na} and ATXII-induced late I_{Na} by resveratrol ($n=5$–11). (**c**) Representative recombinant I_{Na} 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). (**d**) Concentration–response curves for the block of peak I_{Na} and ATXII-induced late I_{Na} by quercetin ($n=4$).

In order to further test the effects of resveratrol and quercetin on late I_{Na}, we used the toxin, *Anemonia sulcata* toxin II (ATXII) (5 nM), to selectively induce a noninactivating late I_{Na} in tsA201 cells expressing wild-type $Na_V1.5$ (Figure 5a and c). At 50 ms, ATXII induced a 20-fold increase in late I_{Na} that was inhibited by resveratrol with an IC_{50} of 26 ± 3.0 μM. (Figure 5b), a threefold more potent block than that of peak I_{Na} (IC_{50} of 77 ± 8.2 μM) (Figure 5b). In contrast to the results found using the R1623Q mutant, quercetin showed a significant difference between its effect on late I_{Na} (25 ± 3.2 μM) and peak I_{Na} (14 ± 2.1 μM, $P<0.05$) (Figure 5d) when late I_{Na} was induced with ATXII.

Myocyte contractility and calcium handling

The studies of resveratrol on recombinant $Na_V1.5$ channels suggest that some of its cardioprotective effects documented in native systems may involve inhibition of I_{Na}, particularly late I_{Na}. In order to test the ability of resveratrol to reduce myocardial dysfunction, we used ATXII 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 (Ravens and Himmel, 1999).

After 5 min of application, 5 nM ATXII increased diastolic calcium (Figure 6a), but when resveratrol (50 μM) was present at the beginning of the experiment, the ATXII-induced elevation in diastolic calcium was prevented. Subsequent removal of resveratrol allowed ATXII to elevate diastolic calcium again (Figure 6b and d). Resveratrol was also found to reverse the effects of ATXII on diastolic calcium: after

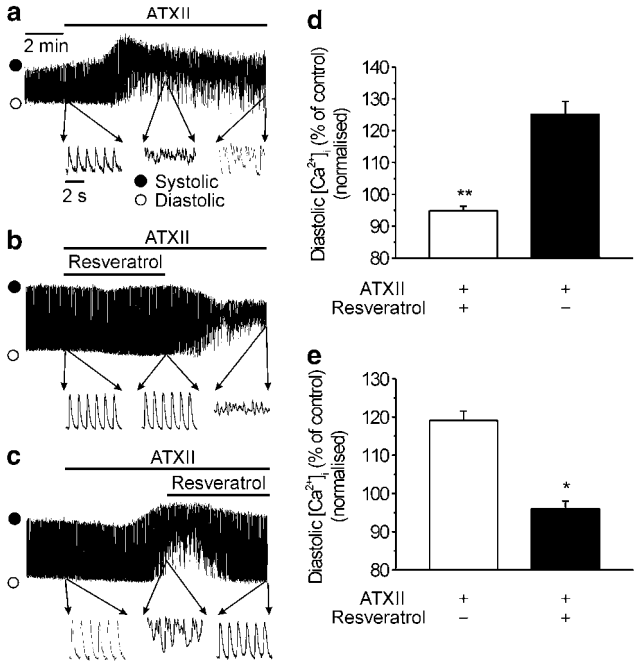

Figure 6 (**a–c**) Representative recordings of calcium green-1AM fluorescence during a 2 min control period followed by a 10 min treatment period. Expanded traces show calcium transients at the 2, 7 and 12 min time points. (**a**) Calcium transient recording in which the 2 min control period is followed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

5 min treatment with ATXII, addition of resveratrol reduced diastolic calcium to control values (Figure 6c and e). The application of resveratrol alone to cardiomyocytes had no effect on the amplitude (91±6% of control) nor on the systolic (96±2% of control) or diastolic (94±4% of control) levels of the calcium transient (results not shown, $n=3$).

While large sustained elevations in intracellular calcium eventually lead to myocyte hypercontracture, this is usually preceded by alterations in contractile function. After-depolarizations generated by sustained sodium levels or excessive calcium levels may be manifested as premature contractions. Accordingly, we measured the effects of ATXII and resveratrol on the contractile behaviour of field-stimulated myocytes. ATXII (3 nM)-induced dysfunction in cardiomyocyte contractility about 140 s after addition (Figures 7a and c). Application of 100 μM resveratrol during the first 5 min of ATXII treatment delayed the initiation of contractile dysfunction threefold (Figures 7b and c). Analysis of the frequency of abnormal contractions that exhibited premature peaks indicative of after-depolarizations revealed that in the presence of ATXII, resveratrol abolished the occurrence of these abnormal contractions (Figure 7d).

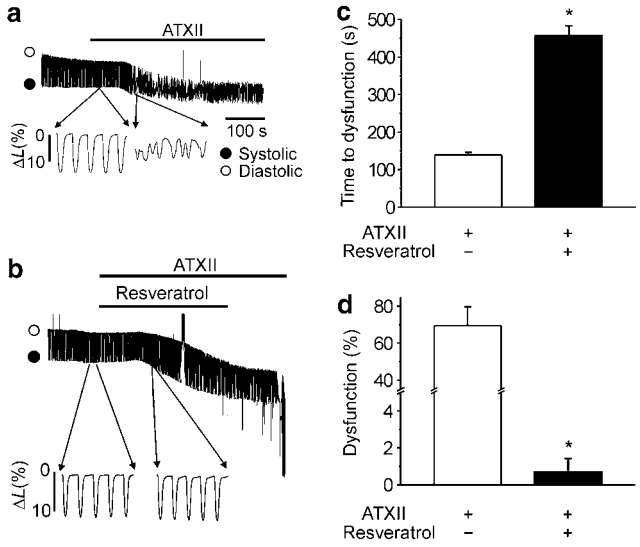

Figure 7 (**a**, **b**) Representative recordings of cardiomyocyte shortening. Expanded traces show contractility at the 2 and 4 min time points. (**a**) 3 nM ATXII produces contractile dysfunction. (**b**) Contractile dysfunction occurs later and to a lesser extent in the presence of both 3 nM ATXII 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 as measured by the occurrence of abnormal contractions; with the addition of 100 μM resveratrol, a significantly smaller incidence of abnormal contractions is observed; $^{*}P<0.001$ ($n=7$ in all groups).

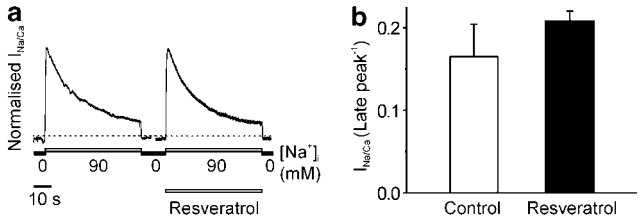

Figure 8 (**a**) Representative traces of recombinant NCX1.1 current ($I_{Na/Ca}$) in the presence or absence of 50 μM resveratrol. (**b**) The ratio of late to peak $I_{Na/Ca}$ in the presence of 50 μM resveratrol ($n=3$) is not significantly different from control ($n=3$). $P>0.05$.

Role of sodium/calcium exchanger

Increases in late I_{Na} lead to elevated intracellular sodium that is thought to favour reverse-mode sodium/calcium exchange (NCX) and the subsequent influx of calcium into myocytes (Ravens and Himmel, 1999). Therefore, a plausible alternative explanation for the effects of resveratrol may involve a direct inhibition of reverse-mode NCX. In order to test this experimentally, we expressed recombinant rat NCX1.1 in tsa201 cells and measured $I_{Na/Ca}$ in the absence and presence of resveratrol (Figure 8a). Resveratrol, at the concentration (50 μM) shown to reduce calcium loading in myocytes, had no significant effect on reverse-mode NCX (Figure 8b).

Discussion

Grape products contain flavonoids and stilbenes, including catechins, quercetin and resveratrol, which have demon-

strated benefits *in vitro* and *in vivo*. Consumption of red wine (20–30 g alcohol day^{-1}) has been associated with a 40% reduction in risk of coronary heart disease (CHD) and a diet rich in flavonoids (30 mg day^{-1}) has been linked to a 50% decrease in CHD mortality (Renaud and de Lorgeril, 1992). While polyphenols have cardioprotective effects related to their antioxidant properties (Frankel *et al*., 1993; Hung *et al*., 2000; Brito *et al*., 2002), it has emerged in recent years that they may also interact with intracellular and cell-surface proteins, including ion channels, independently of these actions (Li *et al*., 2000; Dobrydneva *et al*., 2002; Wallerath *et al*., 2002; Cao and Li, 2004; Orsini *et al*., 2004; Kim *et al*., 2005). Examples of these include potassium channels (Orsini *et al*., 2004), calcium channels (Dobrydneva *et al*., 2002) and neuronal sodium channels (Kim *et al*., 2005).

In addition, we now show that several grape polyphenols directly inhibit cardiac VGSCs and that this mechanism may contribute to the cardioprotective effects of these polyphenols. Specifically, our data demonstrate that application of red grape extract at a concentration relevant to the diet (15 μg ml^{-1}; Manach *et al*., 2004), significantly inhibits wild-type VGSCs (Figure 1a and b). Three of the most common polyphenols in grape extract are quercetin, catechin and resveratrol (Figure 1c), and these polyphenols, in that order of potency, dose-dependently inhibit I_{Na} (Figure 2e).

We also provide direct evidence that the polyphenol resveratrol may exert some of its effects via inhibition of late I_{Na}. Two models of late I_{Na} were used, differing in their mechanisms: the LQT3 mutant R1623Q introduces an intrinsic change in the structure of the VGSC, uncoupling activation and inactivation (Kambouris *et al*., 1998), and the toxin ATXII, by destabilizing the inactivated state (Chahine *et al*., 1996), allows the VGSC to more readily return to the open state. The latter model may be used to pharmacologically manipulate late I_{Na} in the recombinant system and use these results as the basis for experiments on native myocytes. Resveratrol inhibits late I_{Na} to a $\sim$2-fold greater extent than peak I_{Na} in both models of late I_{Na} (Figures 4d and 5b). In comparison, quercetin also displayed some selectivity in inhibiting late I_{Na} vs peak I_{Na} block, although not as great as resveratrol and only in the ATXII model of late I_{Na} (Figure 4d vs Figure 5b and d). These differences may be attributable to the structural differences between resveratrol and quercetin discussed below.

The ability of resveratrol to preferentially inhibit late I_{Na} in the heart has direct relevance to pathophysiological states such as LQT3, I/R injury and arrhythmias. In LQT3, prolongation of the APD can be attenuated by reduced late I_{Na}, leading to a lower likelihood of after-depolarization formation and subsequent torsade de pointes arrhythmias. The antianginal agent ranolazine exhibits selective inhibition of this current, protecting the heart by preventing APD prolongation (Antzelevitch *et al*., 2004). In the case of I/R injury, increases in intracellular sodium via late I_{Na} and sodium–hydrogen exchanger activity (Lazdunski *et al*., 1985; Van Emous *et al*., 1997; Karmazyn *et al*., 1999) are thought to facilitate calcium overload via reverse-mode $I_{Na/Ca}$, thus a reduction in sodium load via late I_{Na} block by polyphenols may reduce or prevent increases in intracellular calcium, reducing cellular damage in the form of irreversible cellular hypercontracture and the generation of arrhythmias. In either of these situations, frequency-independent inhibition of peak I_{Na} may be ineffective or even detrimental. Our results show that while resveratrol both prevents and reverses increases in diastolic calcium induced by ATXII, a VGSC-specific toxin (Figure 6), produces a three-fold delay in ATXII-induced contractile dysfunction (Figure 7c) and reduces the incidence of abnormal contractions, defined as premature peaks (Figure 7d), it has no effects in the absence of pathophysiological conditions (results not shown) and does not produce a cessation of synchronously stimulated calcium transients or myocyte contractility, indicating that it is acting primarily through late and not peak VGSC inhibition. The observed lack of direct effect of resveratrol on reverse-mode $I_{Na/Ca}$ (Figure 8) further 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 reduced reverse-mode $I_{Na/Ca}$. This does not exclude an additional role for L-type calcium channel inhibition (Liew *et al*., 2005), which could explain discrepancies between the effect of resveratrol on I_{Na} and on calcium handling and contractility. It is plausible that other ion channels may also be modulated by these polyphenols, which should be further characterized in future studies.

VGSC blockers, like polyphenols, have a substituted aromatic ring as a common structural feature (Figure 1c). It is thought to be the moiety responsible for binding to their target site, a cluster of hydrophobic residues at the interface of four segments lining the VGSC pore (Ragsdale *et al*., 1994; Haeseler *et al*., 2001; Yarov-Yarovoy *et al*., 2002). In studies in which lidocaine is chemically 'dissected' into its hydrophilic and hydrophobic components (Zamponi and French, 1993; Haeseler and Leuwer, 2002; Haeseler *et al*., 2002), the polar, amine portion exhibits fast, use-dependent open-state block involving changes in channel conductance (Zamponi and French, 1993), while the phenolic part exhibits less frequency-dependence in its inhibition of I_{Na}, in which there are changes in open probability and rapid recovery from inactivated-state block (Zamponi and French, 1993; Haeseler *et al*., 2002). Our finding that resveratrol does not incur use-dependent block of VGSCs further confirms the importance of the charged amine moiety in 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 of an absence of washout indicate this to be unlikely. Furthermore, our data indicate that, as with simple phenolics, voltage-dependence of activation and steady-state availability are not affected by resveratrol or quercetin (Figure 2f and g).

A key finding from this study is the $\sim$2-fold selectivity of resveratrol for late over peak I_{Na} compared to the absence of selectivity in the case of quercetin. An analysis of their structures (Figure 1c) 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 allowing necessary conformational changes for a late current to result (Yarov-Yarovoy *et al*., 2002; Leuwer *et al*., 2004). Differences in the sodium channel residues

involved in coupling of activation to inactivation and in inactivation state stabilization may be responsible for discrepancies between results for resveratrol and quercetin in the two models of late I_{Na}. In addition, our observation that quercetin is a much more effective VGSC inhibitor than catechin (IC_{50} values of 19.4 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.

Additional benefits of resveratrol and other red grape polyphenols may be 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). Changes in redox potential or surface charge may also account for some ionic current block (Bhatnagar *et al*., 1990), therefore 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 1c), demonstrated no effect on I_{Na} (Figure 2d and e), making this an unlikely mechanism in this case.

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 ischaemic recovery (Ray *et al*., 1999), indicating potentially clinically relevant doses. While concentrations of individual polyphenols in the 50 μM range, as used in this study, are unlikely to be reached in human plasma after moderate red wine 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, possibly imparting additive effects on inhibition of I_{Na}.

Our observations that red grape extract and resveratrol are resistant to washout (Figures 1b and 3d) 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 were 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 their aglycone and their glycoside forms in plasma (Vitrac *et al*., 2003; Manach *et al*., 2004); the latter are cleaved by endogenous β-glucosidases to increase polyphenol bioavailability.

In summary, we have demonstrated that several common red grape polyphenols are effective inhibitors of peak and/or late I_{Na}. This mechanism may contribute to the observed protective effects of red grape/wine ingestion on cardiac function during I/R injury. This novel protective mechanism involves improved myocyte calcium handling and contractility that is downstream of inhibition of late I_{Na}. Given the important role that ion channels play in a variety of disease states, further studies on the modulatory effects of grape polyphenols on other types of ion channels are warranted.

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Conflict of interest

The authors state no conflict of interest.

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