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The red wine polyphenol, resveratrol, exerts acute direct actions on guinea-pig ventricular myocytes

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Abstract

Epidemiological evidence suggests that moderate consumption of red wine may be cardioprotective, although the precise mechanism(s) responsible remains poorly understood. We hypothesized that the red wine polyphenol, resveratrol, may exert direct actions on the heart and thus potentially contribute to cardioprotection. We show that resveratrol acutely decreases Ca^{2+} transient amplitude in isolated cardiac myocytes. Intriguingly, resveratrol simultaneously increases cell shortening in half the cells tested, while decreasing shortening in the other half. The former could be attributed to heightened myofilament Ca^{2+} sensitivity. This was no longer observed in myocytes that had been incubated with the oestrogen receptor antagonist, ICI 182,780, suggesting an oestrogen-receptor dependent mechanism of action. In addition, resveratrol significantly decreased action potential duration and the peak L-type Ca^{2+} current. Our findings provide evidence that resveratrol exerts multiple direct actions on cardiac myocytes, the net result of which is no overall change in cell contraction. The clinical significance of these results remains to be determined.

Keywords: Cardiac myocyte; Cell contraction; Ca^{2+} current; Action potential; Red wine

1. Introduction

Cardiovascular disease remains a leading cause of morbidity and mortality in developed countries. In recent years, the possible cardiovascular benefit of low to moderate consumption of alcoholic beverages, particularly of red wine, has received increasing attention (Rimm et al., 1996; Mukamal et al., 2003; Wollin and Jones, 2001). Epidemiological evidence in support of a cardioprotective role of red wine comes from the observation that the mortality rate from coronary heart disease in France, where red wine is commonly taken with meals, is approximately half that of other Western countries, despite the presence of similar cardiovascular risk factors (Renaud and de Lorgeril, 1992; Tunstall-Pedoe et al., 1999). It has been suggested that resveratrol (*trans*-3,4′,5-trihydroxystilbene), a polyphenolic phytoalexin, found abundantly in grapes and red wine (Bhat et al., 2001), may be the beneficial agent responsible for this so called "French paradox" (Kopp, 1998). Resveratrol is likely to exert potential cardioprotective actions through a variety of mechanisms, including anti-oxidant effects (Cao and Li, 2004), inhibition of platelet aggregation (Pace-Asciak et al., 1995), increased expression and activity of endothelial nitric oxide synthase (Wallerath et al., 2002), inhibition of endothelin-1 synthesis (Khan et al., 2002) and vasorelaxation (Naderali et al., 2000; Chen and Pace-Asciak, 1996). However, the full extent and nature of the cardiovascular effects of resveratrol are yet to be determined and, in particular, its potential actions on the heart are completely unknown.

Resveratrol is structurally very similar to the synthetic oestrogen diethylstilbestrol (4,4′,-dihydroxy-*trans*-α,β-diethylstilbene) and has been demonstrated to interact with the two known oestrogen receptors (α and β) (Gehm et al., 1997; Bowers et al., 2000). Since resveratrol acts as a mixed agonist/antagonist at the oestrogen receptor, it may be classified as a selective oestrogen receptor modulator and

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phytoestrogen (being a plant-derived compound with oestrogenic activity). We have previously demonstrated that the mammalian oestrogen, 17β-oestradiol, and a soy-derived phytoestrogen, genistein, can exert direct actions on single ventricular myocytes including Ca^{2+} channel antagonism (Jiang et al., 1992; Liew et al., 2003).

The purpose of the present study was to determine whether resveratrol directly affects the contractile function of guinea-pig ventricular myocytes and to characterize the mechanisms involved. The main advantages of studying single ventricular myocytes in vitro are that direct cardiac actions of the compound being investigated are ensured, concentrations can be accurately controlled and mechanisms of action elucidated. We demonstrate novel cardiac actions of resveratrol at a physiologically relevant concentration of 1 μM (Wu et al., 2001). Consequently, our findings may be of clinical relevance and highlight an additional action of this widely consumed agent on the cardiovascular system.

2. Methods

2.1. Myocyte isolation

Experiments were performed on single left ventricular myocytes isolated from adult male guinea pigs (550–750 g) by enzymatic digestion as previously described (MacLeod and Harding, 1991). Myocytes were stored in Dulbecco's modified Eagle's medium solution at room temperature. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Studies were carried out on at least 10 different cell preparations.

2.2. Cell loading

Cells were loaded at room temperature with 10 μM of the acetoxymethyl ester form of the Ca^{2+}-sensitive fluorescent dye, indo-1 (Molecular Probes, Eugene, OR, USA) for 25 min. Once loaded, cells were not used for at least another 30 min to allow the intracellular indo-1-AM to be de-esterified.

2.3. Effects of resveratrol on the indo-1 dissociation constant

Before indo-1 could be used to accurately record intracellular Ca^{2+} changes in the presence of resveratrol, we first investigated whether the fluorescent properties of the dye were artefactually altered by resveratrol. The in vitro indo-1 K_d was determined using the cell impermeant indo-1 pentapotassium salt (Molecular Probes, Eugene, OR, USA) and 11 pre-diluted buffers containing precise mixes of K_2EGTA/ CaEGTA (Molecular Probes Ca^{2+} Calibration Buffer Kit#2), giving a free Ca^{2+} concentration of 0 to 39.8 μM. A small aliquot of buffer containing 10 μM indo-1 in the presence and absence of 40 μM resveratrol (the maximum concentration used in our study) was placed onto a glass coverslip and the indo-1 ratio determined. All solutions contained an equal amount of dimethyl sulphoxide (DMSO) solvent (0.05%). We found that resveratrol had no significant effect on the in vitro indo-1 K_d [K_d=283±9 nM in control solution (n=9) and 275±11 nM in the presence of 40 μM resveratrol (n=5); P=not significant using unpaired t test].

2.4. Cell shortening and intracellular Ca^{2+} measurements

Cells were placed in a low volume chamber mounted on the stage of an inverted microscope and superfused with normal Tyrode (containing in mM: NaCl 140, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10, pH adjusted to 7.4 using NaOH), heated to 37 °C. A small amount (4 μl) of mouse laminin (Sigma-Aldrich, UK) was applied to the base of the chamber to aid cell adhesion. Two platinum electrodes, placed on either side of the chamber, were used to field stimulate the myocytes at a rate of 1 Hz. Myocytes were directly visualized using a ×40 oil immersion objective (Nikon) and a single rod shaped cell with clear striations, clean edges and good contractions when stimulated was chosen. Since cells tended to become fixed to the base of the chamber at one end, cell shortening during field stimulation was monitored with a video edge-detector system at the free end. Ultraviolet light (wavelength 365±10 nm) from a 100 W xenon arc lamp (Nikon, Japan) was used to excite the fluorescent dye in the cells and the indo-1 ratio (i.e. ratio of light emitted at wavelengths 405 and 485 nm) was recorded using a pair of photomultiplier tubes as described in Terracciano and MacLeod (1997).

Once steady-state contractions were achieved, resveratrol (Sigma-Aldrich, UK) was added to the superfusate and the new steady-state contractions and indo-1 ratio recorded. Similar experiments were performed in cells that had been incubated with the specific oestrogen receptor antagonist, ICI 182,780 (10 μM, Tocris, UK), for 1 h to determine whether the effects of resveratrol on cell shortening and the Ca^{2+} transient were oestrogen receptor-mediated. We have previously shown that this protocol is effective in inhibiting the oestrogen receptor-mediated actions of other oestrogenic compounds (Liew et al., 2004).

As each cell acted as its own control, changes in cell shortening and the indo-1 ratio produced by resveratrol could be compared with initial values in the same cell in control solution. Therefore, the actual indo-1 ratios, rather than calculated Ca^{2+} concentrations, could be used, with the avoidance of any potential errors resulting from dye compartmentalization during calibration experiments (Spurgeon et al., 1990).

2.5. Electrophysiology

Electrophysiological parameters were measured using an Axoclamp-2B system and pCLAMP software (Axon Instruments, Foster City, CA, USA). Action potentials were elicited under current-clamp using 1 nA pulses (5 or 10 ms duration at 1 Hz) and action potential durations at 50% and 90% repolarization (APD_{50} and APD_{90} respectively) were determined. L-type Ca^{2+} currents were recorded under voltage-clamp (discontinuous switch mode with the switching rate set between 4–6 kHz) using a holding potential of −40 mV. Test pulses of 200 ms duration, ranging from −45 to +50 mV, were imposed and L-type Ca^{2+} currents determined by subtracting the current obtained during cadmium application (100 μM) from the one elicited before cadmium application. High resistance microelectrodes were used to limit dialysis of the cells. These were pulled from borosilicate glass (Clark Electromedical Instruments, Reading, UK) and had resistances between 20–30 MΩ when filled with solution containing: KCl 2 M; EGTA, 0.1 mM; HEPES, 5 mM, pH 7.2. Currents were

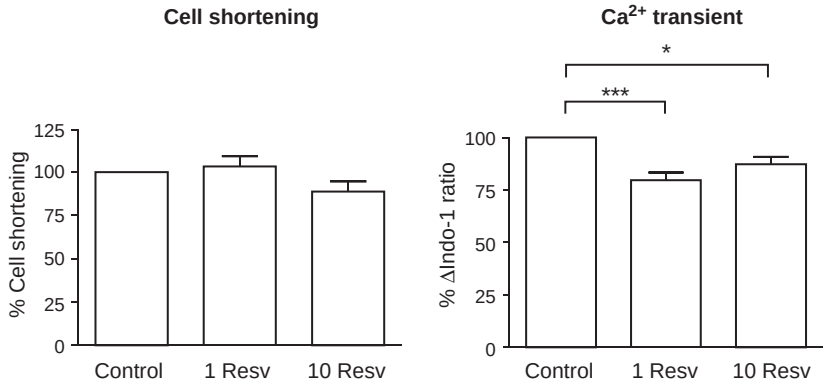

Fig. 1. Effects of resveratrol on cell shortening and Ca^{2+} transient amplitude. Bar graphs showing effects of 1 and 10 μM resveratrol (Resv) on cell shortening (left) and Δindo-1 ratio (right) relative to values in control solution (normal Tyrode), taken as 100%. (*$P<0.05$, ***$P<0.001$; $n=9–14$).

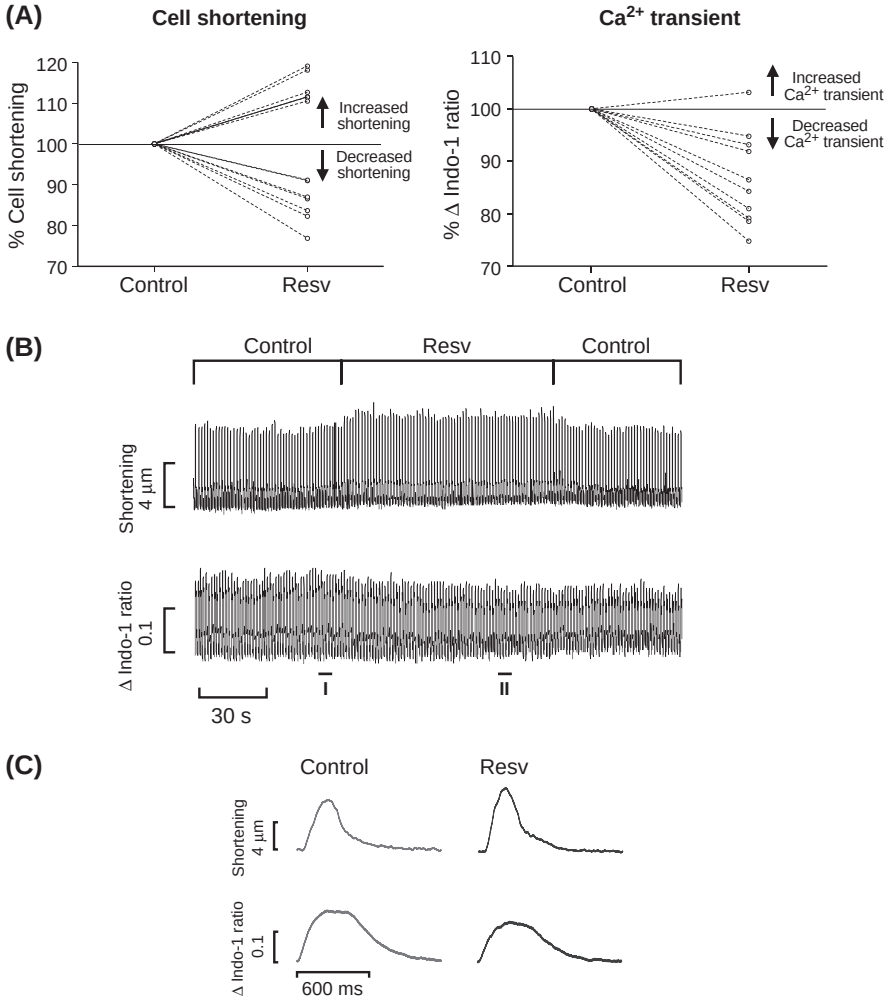

Fig. 2. Response of individual myocytes to 1 μM resveratrol. A) Plot graphs showing effects of 1 μM resveratrol (Resv) on cell shortening (left) and Δindo-1 ratio (right) on each myocyte studied ($n=14$). Control values (measured in normal Tyrode) before the application of resveratrol have been normalised to 100%; values in the presence of resveratrol above this indicate a stimulatory response, and values below this indicate an inhibitory response. B) Sample continuous recording of cell shortening and the Δindo-1 ratio showing the gradual increase in cell shortening in the presence of resveratrol, with little corresponding change in the Δindo-1 ratio. C) Magnified averaged traces of 8 consecutive twitches in parts I and II of the continuous traces in (B) plotted at a faster sweep speed showing twitch and Ca^{2+} transient morphologies.

normalized to cell capacitance in order to account for cells of varying sizes.

2.6. Drugs

All drugs were analytical grade and dissolved in DMSO to make a 100 mM stock solution. The maximum final concentration of DMSO used (0.05%) had no significant effect on the parameters measured.

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. and analyzed using the paired Student's *t* test or one-way analysis of variance (ANOVA) and the Bonferroni post-test. A value of $P<0.05$ was considered significant.

3. Results

3.1. Effects on cell shortening and Ca^{2+} transient amplitude

Resveratrol had no significant overall effect on cell shortening at a concentration of 1 or 10 μM, Fig. 1. However, resveratrol significantly decreased Ca^{2+} transient amplitude at both concentrations (to 79.6 ± 3.8% and 87.3 ± 3.5% of control values, respectively, $P<0.001$ and <0.05). There was no clear concentration–response effect of resveratrol on these two parameters, since effects produced by 1 μM resveratrol were no different from those produced at the higher concentration of 10 μM. The decline in Ca^{2+} transient amplitude occurred quickly over the course of two minutes and was not reversible upon washout with normal Tyrode. We found that many cells became arrhythmic and died in the presence of 40 μM resveratrol ($n=4$). Therefore, it was not possible to perform statistical analyses at this concentration.

Despite no overall change in cell shortening in the presence of 1 μM resveratrol, sub-analysis of the response of individual myocytes revealed a clear dichotomy, with half (7 out of 14 cells) exhibiting increased cell shortening (to 113.6 ± 1.3% of control values, $P<0.05$) and the remainder showing decreased shortening (to 85.5 ± 1.9% of control values, $P<0.001$), Fig. 2A. Interestingly, resveratrol significantly decreased Ca^{2+} transient amplitude in both subsets of cells (to 79.3 ± 5.3% of control values in the stimulated cells, $P<0.01$, and to 79.9 ± 5.8% in the unstimulated cells, $P<0.05$). Fig. 2B shows representative continuous recordings of cell shortening and along with the concurrent indo-1 ratio.

A likely explanation for the above findings is that resveratrol increases myofilament Ca^{2+} sensitivity in the subset of cells that exhibited an increase in cell shortening when exposed to resveratrol. Fig. 3A shows the simultaneous recordings of cell

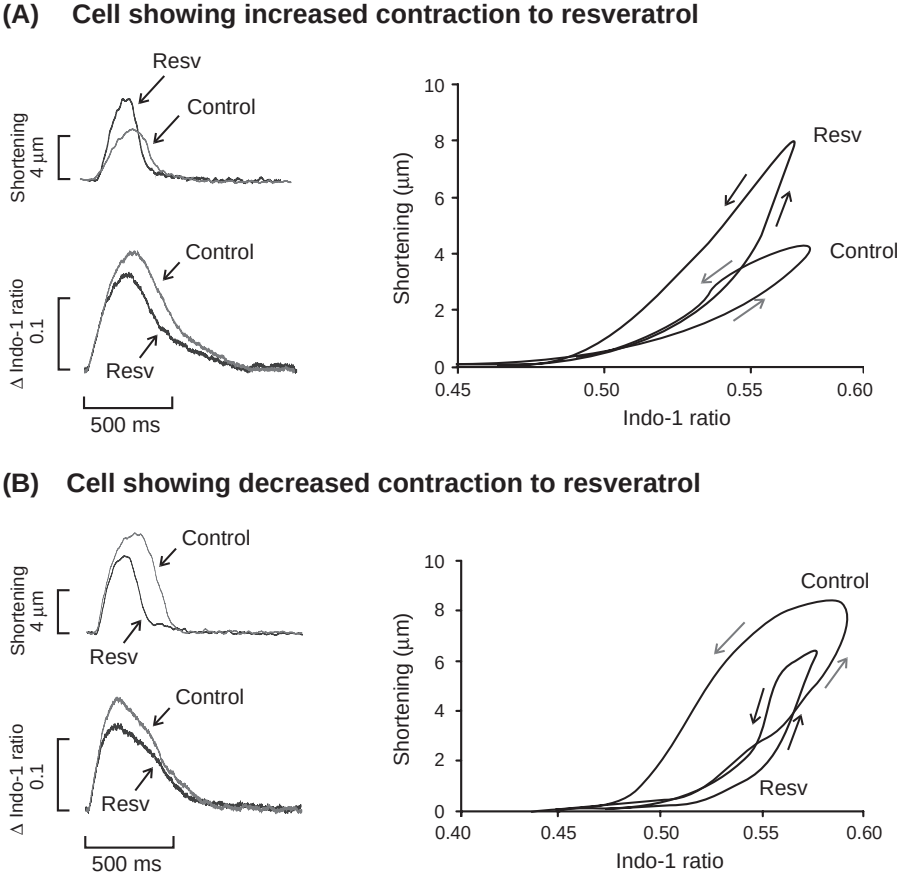

Fig. 3. Increased myofilament Ca^{2+} sensitivity in stimulated cells in the presence of resveratrol. Sample traces of cell shortening and Ca^{2+} transients (averaged from 8 consecutive twitches) taken from a cell showing a stimulatory (A) and inhibitory (B) response to 1 μM resveratrol (Resv). Corresponding phase-plane plots of cell shortening against the indo-1 ratio are shown on the right. Loops proceed in a counter-clockwise direction, with shortening represented by the ascending limb and relaxation by the descending limb.

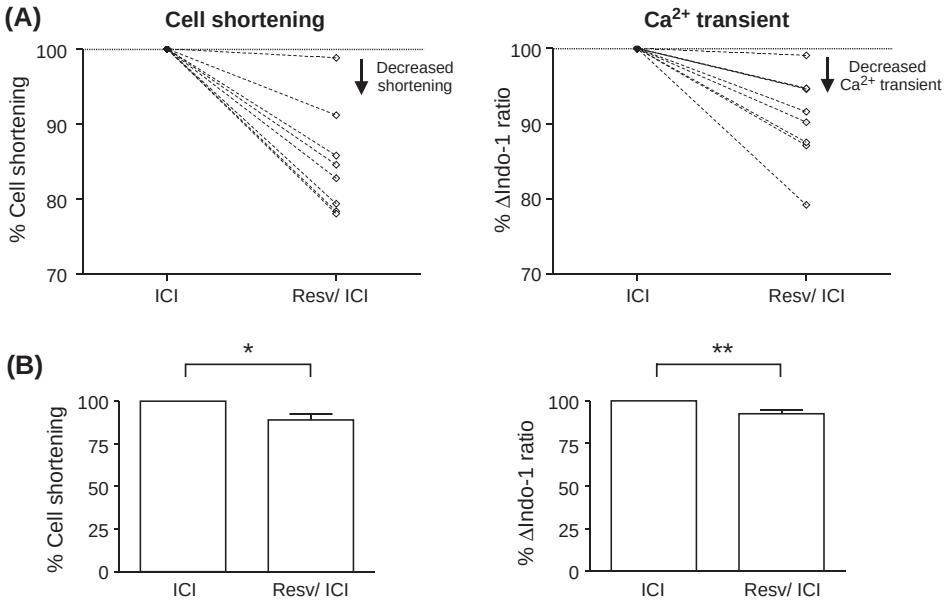

Fig. 4. Effects of resveratrol in cells incubated with ICI 182,780. A) Plot graphs showing effects of 1 μM resveratrol (Resv) on cell shortening (left) and Δindo-1 ratio (right) on cells incubated with ICI 182,780 (ICI). B) Bar graphs showing mean data from (A) relative to control values in the presence of 10 μM ICI 182,780 alone (taken as 100%). ($n=10$, *$P<0.05$, **$P<0.01$).

shortening and the Δindo-1 ratio in a myocyte in which cell shortening was increased and the Ca^{2+} transient decreased in the presence of resveratrol. A qualitative estimate of myofilament Ca^{2+} sensitivity can be obtained by plotting the two parameters against one another (Spurgeon et al., 1992), as shown in the phase-plane plot for that cell. Resveratrol (1 μM) produced a leftward shift in the phase-plane loop, suggesting an increase in myofilament Ca^{2+} sensitivity. In contrast, in cells in which shortening was decreased in the presence of resveratrol, the compound decreased peak values, but otherwise had either no effect on the phase-plane loops or produced a slight rightward shift, as shown in the sample cell of Fig. 3B.

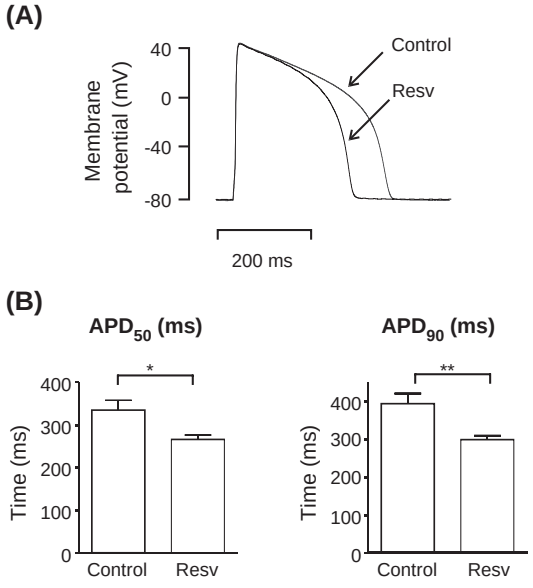

Fig. 5. Effects of resveratrol on action potential duration (APD). A) Representative action potential profiles during steady state stimulation in normal Tyrode (control) and in the presence of 1 μM resveratrol (Resv). B) Bar graphs showing effects of resveratrol on the times-to-50% and 90% repolarization (APD_{50} and APD_{90} respectively). ($n=9$, *$P<0.05$, **$P<0.01$ compared with equivalent times in normal Tyrode).

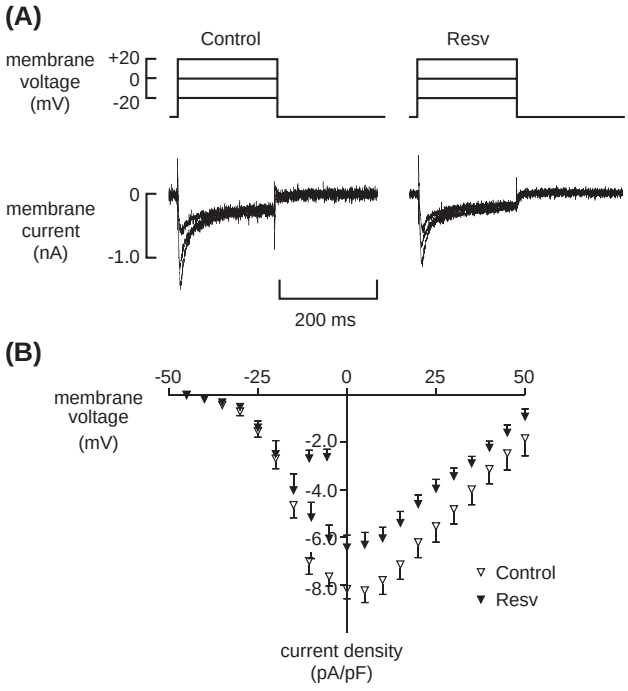

Fig. 6. Effects of resveratrol on the peak L-type Ca^{2+} current ($I_{Ca,L}$). A) Sample recordings showing $I_{Ca,L}$ (following cadmium-subtraction) elicited at three depolarizing potentials in normal Tyrode (control) and in the presence of 1 μM resveratrol (Resv) from a holding potential of −40 mV. B) Current–voltage relationships in the presence and absence of resveratrol. Means ± SEM are plotted, $n=13$.

3.2. Effects in the presence of ICI 182,780

The process of myocyte incubation with 10 μM ICI 182,780 did not significantly alter cell shortening or Ca^{2+} transient parameters ($n=7$, results not shown). We found that 1 μM resveratrol no longer increased cell shortening in any cell following incubation with ICI 182,780 (Fig. 4A). In fact, resveratrol (in the continuing presence of ICI 182,780) significantly decreased overall cell shortening and Ca^{2+} transient amplitude in ICI 182,780 incubated cells to 89±3% ($n=10$, $P<0.05$) and 92±2% ($n=10$, $P<0.01$) of control values, respectively (Fig. 4B).

3.3. Electrophysiological actions

Fig. 5A illustrates the effects of resveratrol on action potential waveform. Resveratrol significantly decreased APD_{50} from 335±23 to 266±10 ms ($n=9$, $P<0.05$) and APD_{90} from 395±27 to 299±10 ms ($P<0.01$). There was no significant change in the resting membrane potential.

Resveratrol decreased L-type Ca^{2+} current over the range of potentials tested without altering the shape of the current–voltage relationship, Fig. 6. Peak L-type Ca^{2+} current declined from −8.2±0.5 pA/pF to −6.3±0.5 pA/pF ($n=13$, $P<0.05$) in the presence of resveratrol and occurred over a similar timescale to that observed with Ca^{2+} transient amplitude.

4. Discussion

The principal finding of the present study is that the red wine polyphenol, resveratrol, exerts direct actions on isolated cardiac myocytes at a physiologically relevant concentration of 1 μM. During cardiac excitation–contraction coupling, Ca^{2+} enters the cell through surface membrane L-type Ca^{2+} channels following membrane depolarization and triggers further release of Ca^{2+} from intracellular Ca^{2+} stores. This culminates in a large rise in free intracellular Ca^{2+} (the Ca^{2+} transient) which subsequently produces cell contraction through interactions with the contractile apparatus (Bers, 2002). We have shown that resveratrol acts as a Ca^{2+} channel antagonist and consequently decreases the Ca^{2+} transient amplitude. This would normally be expected to result in decreased cell shortening. Intriguingly, however, we provide evidence that resveratrol increases cell shortening when the Ca transient decreases. This implies that the polyphenol increases myofilament Ca^{2+} sensitivity in a subset of cells.

In cells that had been incubated with ICI 182,780, resveratrol did not increase cell shortening suggesting that the stimulatory effect on myofilament Ca^{2+} sensitivity is mediated through the oestrogen receptor. Conversely, the fact that resveratrol continued to decrease Ca^{2+} transient amplitude in ICI 182,780 incubated cells suggests an additional oestrogen receptor-independent mechanism of action. It is therefore likely that resveratrol exerts multiple actions on isolated cardiac myocytes, some of which are oestrogen receptor-dependent and others oestrogen receptor-independent. This is consistent with other reports of mixed effects of resveratrol on the cardiovascular system (Mizutani et al., 2000; El Mowafy and White, 1999; Wallerath et al., 2002). Resveratrol binds oestrogen receptor α and oestrogen receptor β with comparable affinity, but with a 7000-fold lower affinity than 17β-oestradiol (Bowers et al., 2000). However, since physiological concentrations of 17β-oestradiol are in the nanomolar range, micromolar concentrations of resveratrol are likely to produce significant interactions in vivo with those of 17β-oestradiol.

Cardiac myocytes used in the present study were obtained from the left ventricle with no attempt made to further subdivide the muscle into endo-, mid- and epicardial layers. Regional differences in myocyte function across the ventricular wall, including differences in myofilament Ca^{2+} sensitivity, are known to exist (Chamunorwa and O'Neill, 1995). The stimulatory response exhibited by half the cells to resveratrol may therefore represent the response of a specific layer of the myocardium. Alternatively, since the stimulatory action of resveratrol appears to be mediated via the oestrogen receptor, the heterogeneous action of resveratrol on cell shortening may be related to differential expression of surface oestrogen receptors (α, β or both) between myocytes. Although cardiac myocytes have been demonstrated to express functional oestrogen receptors (Grohe et al., 1997), the relative expression of oestrogen receptors between cells is unknown. In support of this hypothesis, however, is the finding of heterogeneous surface binding of fluorescently labelled bovine serum albumin conjugated 17β-oestradiol in GH_3/B6 rat pituitary tumour cells (Pappas et al., 1995). Clearly, further work is required to separate out these possibilities. It is interesting to note that the heterogeneous response of myocytes to resveratrol presented here is very similar to our previous finding with the phytoestrogen genistein (Liew et al., 2003), suggesting a common effect among some phytoestrogens in increasing myofilament Ca^{2+} sensitivity.

The shortened action potential duration observed in the presence of resveratrol may solely be a function of L-type Ca^{2+} current inhibition and a decreased amplitude. However, stimulatory effects on repolarizing K^+ currents, which can also shorten action potential duration, cannot be excluded. We are unaware of any studies investigating the actions of resveratrol on K^+ channels in the heart, although resveratrol has been demonstrated to stimulate Ca^{2+}-activated K^+ currents in vascular endothelial cells (Li et al., 2000).

Resveratrol has been shown to be present in the plasma of rats soon after oral administration of red wine (Bertelli et al., 1996). Furthermore, prolonged administration of red wine leads to increased amounts of resveratrol in different tissues, particularly the heart and liver (Bertelli et al., 1998). The findings presented here are therefore likely to be physiologically relevant in vivo. However, it is worth noting that red wine contains many biologically active components,

which can exert actions of their own and/or interact with those of resveratrol. In particular, physiologically relevant concentrations of ethanol have been shown to directly suppress cardiac myocyte contractility (Delbridge et al., 2000) and decrease myofilament Ca^{2+} sensitivity (Danziger et al., 1991). Such actions are thus likely to interfere with those of resveratrol and the net effect will depend on the relative concentrations and potencies of the various bioactive compounds.

In conclusion, we have provided evidence that resveratrol exerts multiple direct actions on cardiac myocytes including Ca^{2+} channel antagonism. The net result, however, is that there is no overall change in cell contraction. Whether these in vitro data translate into clinical benefit is a subject for future functional and/or clinical studies that take into account the actions of other agents present in red wine which may interact with those of resveratrol.

Acknowledgements

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