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Research article

Effects of superoxide donor menadione in adult Rat myocardium are associated with increased diastolic intracellular calcium

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Superoxide anions have been associated with many aspects of cardiovascular disease. Menadione is a superoxide anion donor that alters the heart's electrical and mechanical functions. The aim of this study was to demonstrate simultaneous changes in intracellular Ca^{2+} ($[Ca^{2+}]i$) and mechanical activity in intact adult cardiac myocytes, and mechanical activity and electrical activity in isolated whole hearts in order to provide greater insight into the mechanisms associated with the detrimental effects of menadione on the myocardium. Isolated hearts from adult male Wistar rats ($n = 11$, 200–250 g) were Langendorff perfused at 38°C with a Krebs–Henseleit solution. A saline-filled balloon was placed in the left ventricle (LV) in order to measure diastolic and developed pressure. Monophasic action potentials were simultaneously recorded from the epicardial surface. External stimulation at 5 Hz and intrinsic pacing were used throughout a 10 min control period and 30 min exposure to 50 μM menadione. Single LV myocytes ($n = 7$ from $n = 4$ animals) were loaded with the Ca_{2+}-indicator Fura4-AM, stimulated at 1 Hz and exposed to 50 μM menadione. Myocyte length was simultaneously measured with $[Ca^{2+}]i$ using a video edge detection system. In isolated hearts, exposure to menadione significantly decreased contractility and action potential duration (with a similar time course); intrinsic heart rate and rhythmicity. Diastolic pressure was significantly increased. In single adult myocytes, menadione caused a significant increase in diastolic $[Ca^{2+}]i$ and a decrease in resting cell length and led to spontaneous release of $[Ca^{2+}]i$. We conclude that the effects of menadione upon electrical and mechanical activity of the heart are at least in part a consequence of dysregulation of $[Ca^{2+}]i$ handling and the subsequent increase in diastolic $[Ca^{2+}]$ alterations in $[Ca^{2+}]i$ are consistent with the generation of delayed after depolarization arrhythmias.

Key words: menadione, calcium, rat, superoxide, contractility, electrophysiology

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Introduction

Cardiovascular disease is a major contributor to mortality and morbidity throughout the world (Roger *et al.*, 2012). In the case of acute ischaemic events current treatment guidelines prioritize the rapid restoration of circulatory function to reverse cardiomyocyte injury and limit cell death, thus significantly reducing cardiac mortality and morbidity (Pollack, Antman, and Hollander, 2008). However, the advent of cardiac thrombolysis through the use of agents such as streptokinase and percutaneous coronary interventions has led to the characterization of a paradoxical phenomenon known as 'reperfusion injury'. This is believed to occur as a result of the rapid production of reactive oxygen species (ROS), which overwhelm the endogenous antioxidant systems leading to cell death via necrosis and apoptotic pathways. The superoxide anion in particular is believed to have a critical role as a mediator of post-ischaemic contractile dysfunction, dysrhythmias and chronic cardiovascular disease (Kevin, Novalija and Stowe, 2005).

Menadione is a potent superoxide donor (Choi *et al.*, 2005) that has a negative inotropic effect on isolated hearts, which, it has been suggested, is linked to intracellular Ca^{2+} ($[Ca^{2+}]i$) regulation (Anderson and Dutta, 1991). In addition, it has been demonstrated that menadione alters the electrical response of cardiac tissue preparations (Choi *et al.*, 2005; Ha *et al.*, 2007). However, the simultaneous measurement of the mechanical and electrical effects of menadione has not previously been reported, nor has its effects on $[Ca^{2+}]$i transients in intact adult myocytes been measured. The objective was to test the hypothesis that the mechanical and electrical effects of the superoxide anion donor menadione can be explained by the presence of dysfunctional $[Ca^{2+}]i$ regulation.

Materials and Methods

Isolated whole hearts

Adult male Wistar rats ($n = 13$, 200–250 g) were killed by stunning and cervical dislocation in accordance with the UK Home Office regulations (Animal [Scientific Procedures] Act 1986). Hearts were isolated, weighed and Langendorff perfused (Stones *et al.*, 2009) with a Krebs–Henseleit (K–H) solution at 38°C at a constant flow rate of 7 ml min^{-1} g^{-1} heart weight. A saline-filled balloon, connected to a pressure transducer, was placed in the left ventricle (LV) via the dissected left atrium to measure diastolic and developed pressure (DP). The balloon was inflated (typically to a volume of 0.1 ml) until diastolic pressure began to register and transient systolic pressures were visible. Monophasic action potentials (MAPs) were simultaneously recorded from the epicardial surface of the LV (Benoist *et al.*, 2011). Alternating, 5 min periods of external stimulation, delivered via platinum contact electrodes at a frequency of 5 Hz and intrinsic pacing (no external stimulation), were used throughout a 10 min control and 30 min exposure to 50-µM menadione (Sigma, Aldrich) followed by a return to menadione-free solution. Alternatively, after 10 min control solution, hearts ($n = 2$) were exposed to glibenclamide (a blocker of ATP-modulated potassium current, I_{KATP}) for 10 min followed by glibenclamide plus menadione for 30 min. Pressure values and rates of pressure development and MAP durations were measured with Lab Chart 7 software (ADInstruments, Australia).

Single LV myocytes

LV myocytes were isolated from $n = 4$ adult Wistar rat hearts as described previously by McCrossan, Billeter and White (2004). Myocytes selected for study were quiescent when not stimulated and had clear and regular striations. Myocytes were loaded with the Ca^{2+}-indicator Fura4-AM (2 µM for 20 min) stimulated at 1 Hz and exposed to 50 µM menadione ($n = 7$). Cells were alternately excited by light at 340 and 380 nm (optoscan monochromator, Cairn Research, UK) and the ratio of emitted light at 510 nm was our index of $[Ca^{2+}]i$. Myocyte length was simultaneously measured using a video edge detection system (Crescent Electronics, Sandy, UT, USA). $[Ca^{2+}]i$ transients and cell shortening were analysed with pClamp 9 (Axon Instruments).

Solutions

The K–H solution contained (in mM) NaCl 118.5; $NaHCO_3$ 25.0; KCl 4.2; KH_2PO_4 1.2 mM; $MgSO_4 7H_2O$ 1.2; glucose 11.1 and $CaCl_2$ 2.0. Menadione was dissolved in methanol to make a 50 mM stock solution which was added to K–H solution to give a final concentration of 50 µM menadione and 0.1% methanol. Glibenclamide, at a final concentration of 50 µM, was dissolved in 0.1% methanol. Exposure to 0.1% methanol in K–H solution had no statistically significant effects on isolated whole hearts or single myocytes. The effects of menadione were not reversible on removal of the agent.

Statistical analysis

Statistical significance was tested using one-way repeated measures ANOVA (RMANOVA) unless stated otherwise, P values < 0.05 were regarded as significant. Data are expressed as mean ± SEM.

Results

Isolated whole hearts

Menadione caused a significant increase in diastolic pressure approximately 10 min after exposure compared to vehicle alone (Fig. 1). The end-diastolic pressure (EDP) rose from 19.7 ± 12.84 mmHg, prior to exposure, to 50.7 ± 9.9 mmHg, ($P < 0.001$) after 25 min. As a consequence of this, DP fell from 76.5 ± 15.72 to 17.5 ± 3.0 mmHg over the same time period ($P < 0.001$). The mean changes for EDP and DP are shown in Fig. 2A and B, respectively.

Exposure to menadione for 25 min also significantly reduced the rate of peak pressure development (max dP/dT) (from 4037.0 ± 446.2 to 2254.2 ± 98.0 mmHg s^{-1}, $P < 0.001$) and the peak rate of relaxation (min dP/dT) (from –3126.6 ± 259.0 to –2240.0 ± 36.5 mmHg s^{-1}, $P < 0.001$). The mean changes of max and min dP/dT are shown in Fig. 2C and D, respectively.

MAP durations at 20, 50 and 80% repolarization (APD_{20}, APD_{50}, APD_{80}, respectively) were simultaneously recorded with LV pressure (Fig. 3A). There was a significant reduction in APD_{80} after 25 min exposure to menadione (Fig. 3B and C).

In the absence of external stimulation, menadione affected the rate and rhythmicity of intrinsic pacing (Fig. 4A). Heart rate was significantly reduced after 20 min of exposure (Fig. 4B, $P < 0.05$, paired t-test). There was a significant decrease in rhythmicity, indicated by an increase in the standard deviation of beat-to-beat periodicity (an established index of rhythmicity, Fig. 4C, $P < 0.05$, Wilcoxon signed rank test).

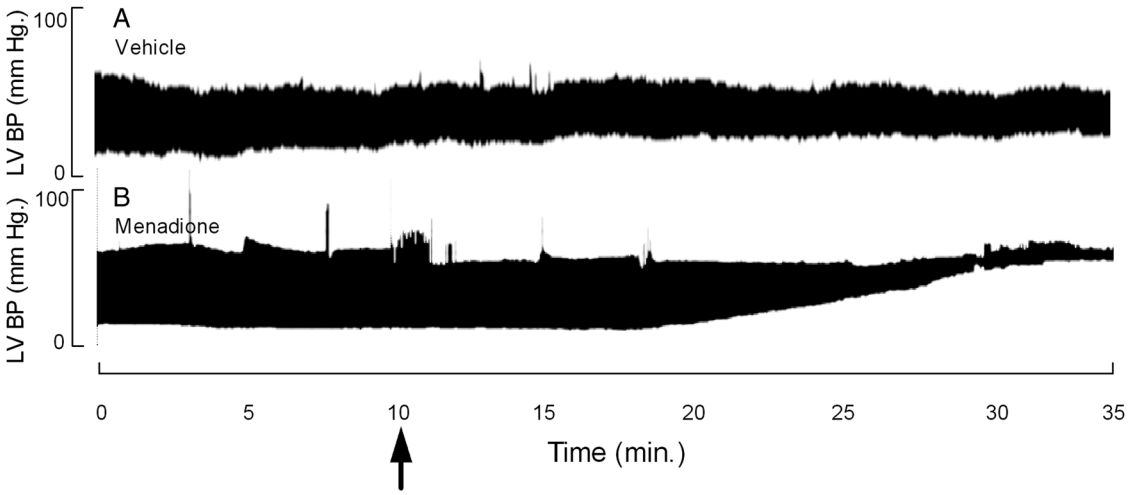

Figure 1. Representative trace of LV pressure before and after exposure to vehicle (**A**) or 50 μM menadione (**B**). Arrow indicates addition of agents.

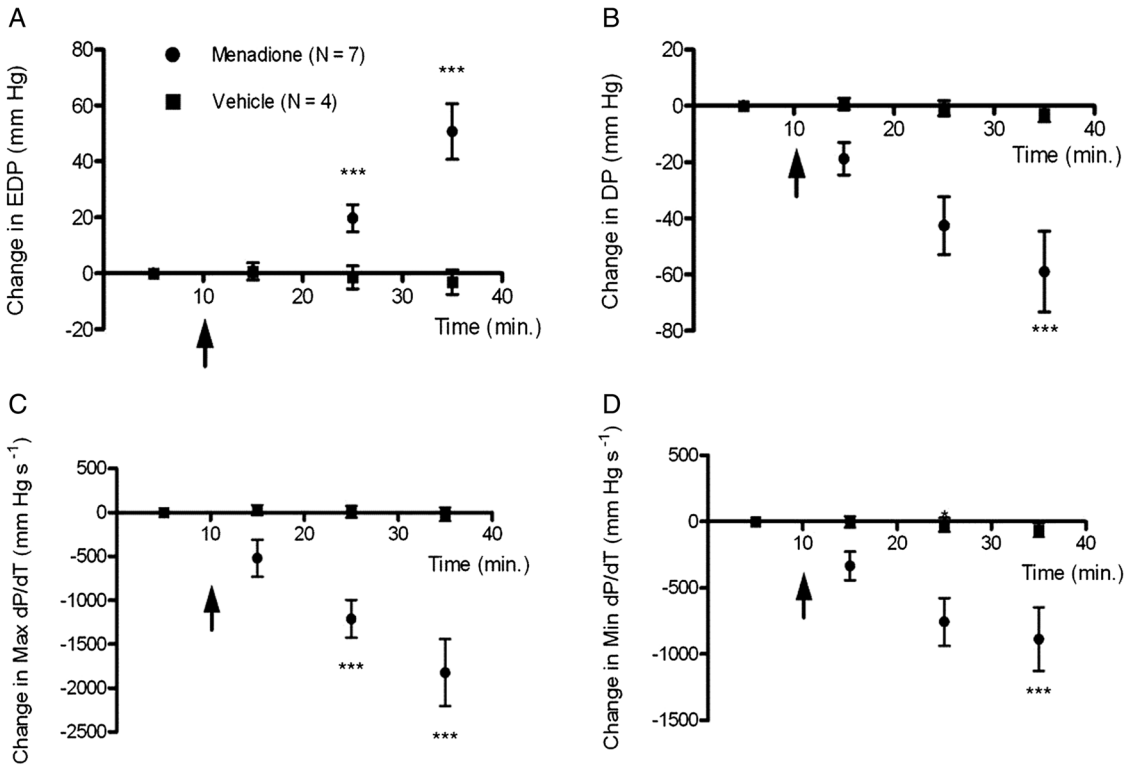

Figure 2. Mean changes in (**A**) EDP, (**B**) DP, (**C**) maximum rate of pressure development (contraction) and (**D**) minimum rate of pressure development (relaxation). Arrow denotes addition of vehicle or 50 μM menadione. ***$P < 0.001$ vs. pre-drug, 1-way RMANOVA, $n = 7$ hearts.

To test whether action potential duration (APD) shortening was dependent upon the activation of I_{KATP}, hearts ($n = 2$) were exposed to the I_{KATP} blocker glibenclamide. This did not prevent the mechanical responses or APD_{80} shortening upon exposure to menadione (APD_{80} shortening 59 and 38%; increase in EDP 52 and 8 mmHg; decrease in DP 27 and 70 mmHg; decrease in max dP/dt 1641 and 1856 mm Hg s^{-1}; decrease in min dP/dT 859 and 1216 mm Hg s^{-1}) these observations are consistent with the changes in these parameters reported in Figs 2 and 3.

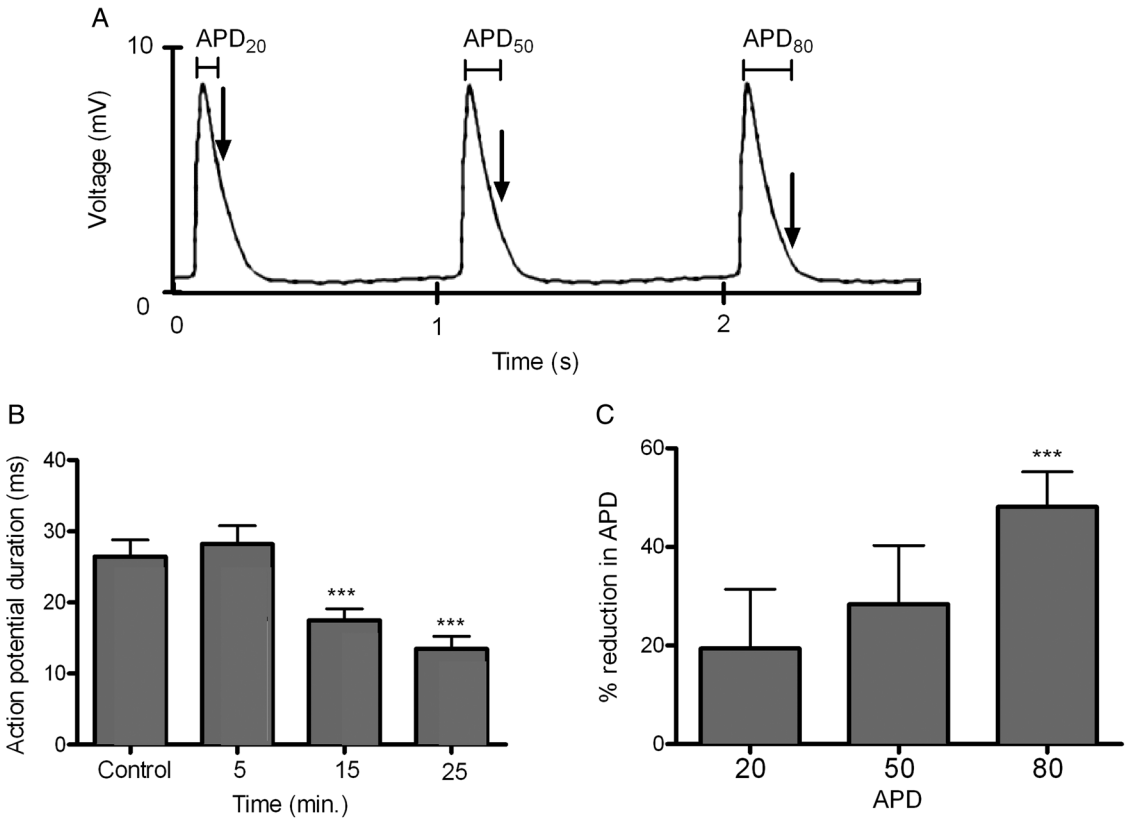

Figure 3. MAP duration following exposure to menadione. (**A**) Representative MAP traces illustrating APD_{20}, APD_{50} and APD_{80}. (**B**) APD_{80} prior to (control) and during exposure to menadione. (**C**) Mean percentage change in MAP duration after 25 min exposure to menadione for APD_{20}, APD_{50} and APD_{80}. ***$P < 0.001$, 1-way RM ANOVA vs. pre-exposure, $n = 7$ hearts.

Single LV myocytes

The effect of menadione on $[Ca^{2+}]i$ handling was tested in single myocytes (Fig. 5A). Exposure to 50 µM menadione caused a significant increase in diastolic calcium (Fig. 5B, $P < 0.05$). There was also a significant reduction in resting cell length (Fig. 5C, $P < 0.05$). Exposure to menadione caused the generation of spontaneous oscillations of $[Ca^{2+}]i$ and cell shortening (Fig. 5A). These effects were not seen in four cells exposed to vehicle alone.

Discussion

The negative inotropic effect of menadione

A novel aspect of our study is that while previous studies have predicted the diastolic contracture provoked by menadione is associated with a rise in diastolic $[Ca^{2+}]i$, our observation in intact, adult myocytes is the first to show directly a simultaneous increase in these parameters. Menadione has been shown to have both positive and negative inotropic effects depending upon concentration (Floreani, Santi, and Carpenedo, 1989). We used a concentration designed to provoke the negative inotropic effect associated with ROS-induced injury (Floreani, Santi, and Carpenedo, 1989; Anderson and Dutta, 1991). We observed decreased DP, as a result of increased diastolic pressure, rather than decreased systolic pressure, consistent with the findings of Anderson and Dutta (1991). Our data provide evidence that exposure to menadione results in a reduction in ventricular DP as a consequence of increased diastolic $[Ca^{2+}]i$, as observed in single myocytes. In this respect, it is interesting to note that the rise in the diastolic pressure in whole hearts (Figs 1 and 2) had a similar time course to the rise in diastolic $[Ca^{2+}]i$ and fall in resting cell length (Fig. 5).

In a previous study, a reduction in myocyte contractility induced by exposure to an alternative ROS donor, H_2O_2, was attributed to a fall in myofilament Ca^{2+} sensitivity (Luo *et al.*, 2006). However, in our study a fall in myofilament Ca^{2+} sensitivity is not consistent with the maintained systolic pressure or a decreased rate of relaxation. Alternatively, in isolated sarcoplasmic reticular (SR) preparations, menadione has been reported to inhibit the function of the SR Ca^{2+}-uptake pump (SERCA) (Floreani and Carpenedo, 1989; Floreani, Santi, and Carpenedo, 1989; Shneyvays *et al.*, 2005). This inhibition could explain the increased level of diastolic $[Ca^{2+}]i$; decreased contractility and decreased rate of relaxation.

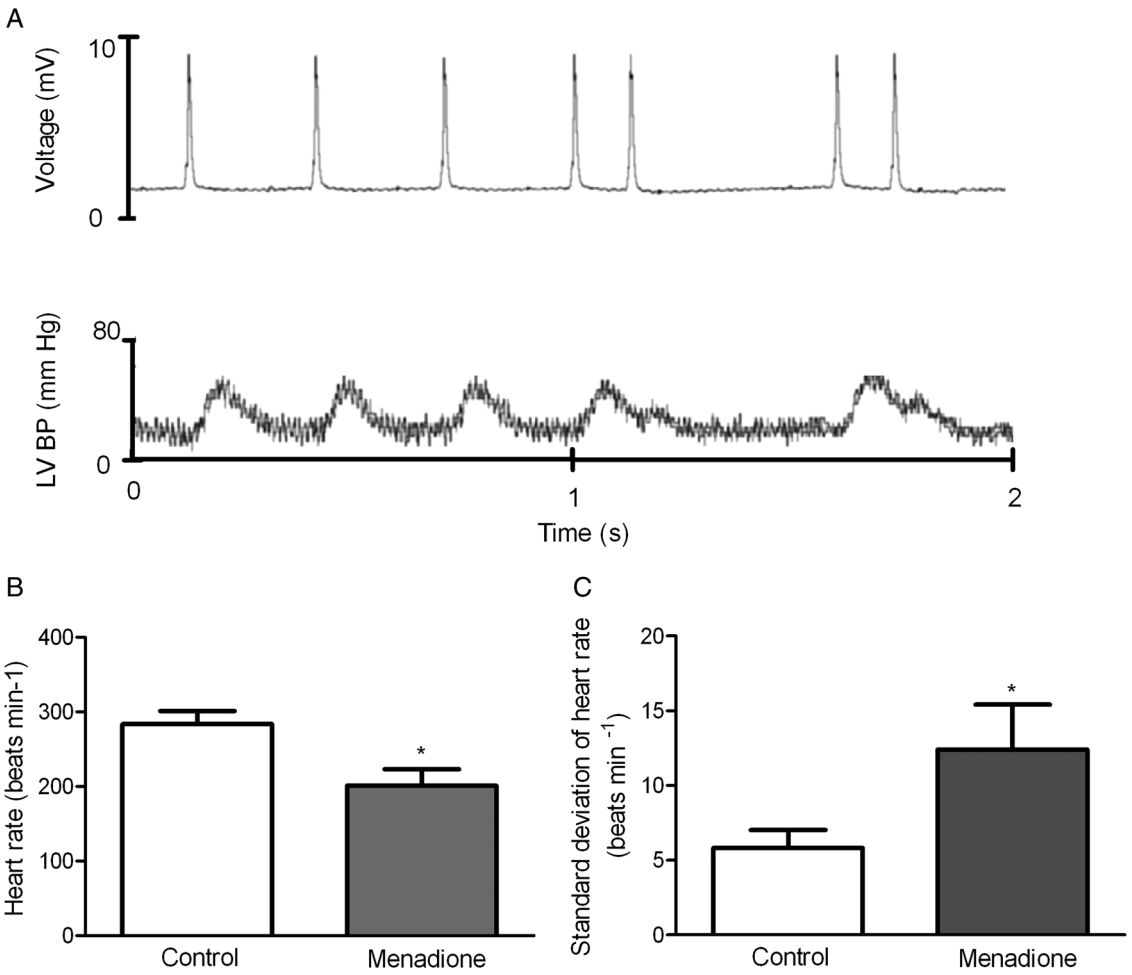

Figure 4. (**A**) Representative, simultaneous recording of LV MAPs (upper trace) and LV pressure (lower trace) in a heart exposed to 50 µM menadione for 20 min, note the dysrhythmic behaviour. (**B**) Intrinsic heart rate and (**C**) standard deviation of heart rate (beat-to-beat periodicity), an index of rhythmicity, before and after 20 min exposure to menadione, $n = 7$ hearts, $^*P < 0.05$; B, paired *t*-test; C, Wilcoxon signed rank test.

Similar findings have been reported in rat ventricular myocytes using H_2O_2 (Greensmith, Eisner and Nirmalan, 2010). Exposure to free radicals has been shown to reduce the activity of Na^+ and K^+-ATPase (Kim and Akera, 1987; Matsuoka, Kato and Kako, 1990). It is therefore possible that cellular loading of Na^+ could also contribute to Ca^{2+} overload via activation of the Na^+–Ca^{2+} exchanger (Matsuura and Shattock, 1991).

Previously, it has been shown that the reintroduction of molecular oxygen to ischaemic myocardium results in the production of oxygen-free radicals (Bolli *et al.*, 1989). Contraction band necrosis has been documented in histological sections of human LV tissue following reperfusion after myocardial infarction and fibrinolysis (Verma *et al.*, 2002). It is therefore interesting that we observed myocyte death (e.g. Fig. 5A) in a number of the myocytes exposed to menadione.

The effect of menadione on APD and heart rate

Menadione reduced the duration of APD_{80} in the LV of isolated whole rat hearts. These findings are consistent with the findings of Choi *et al.* (2005) and Ha *et al.* (2007). However, Barrington, Meier and Weglicki (1988) provided evidence that H_2O_2 prolonged the APD of canine ventricular myocytes and Cerbai *et al.* (1991) have shown that dihydroxyfumaric acid (DHF), a general donor of ROS species, prolongs the APD of guinea pig ventricular myocytes. These study differences may arise from the varying effects of ROS species and or related to differences in species studied or preparation, e.g. single cells vs. multicellular preparations.

Menadione also had a negative chronotropic effect on isolated whole hearts and produced dysrhythmia. Cerbai *et al.* (1991) have shown that DHF causes guinea pig ventricular

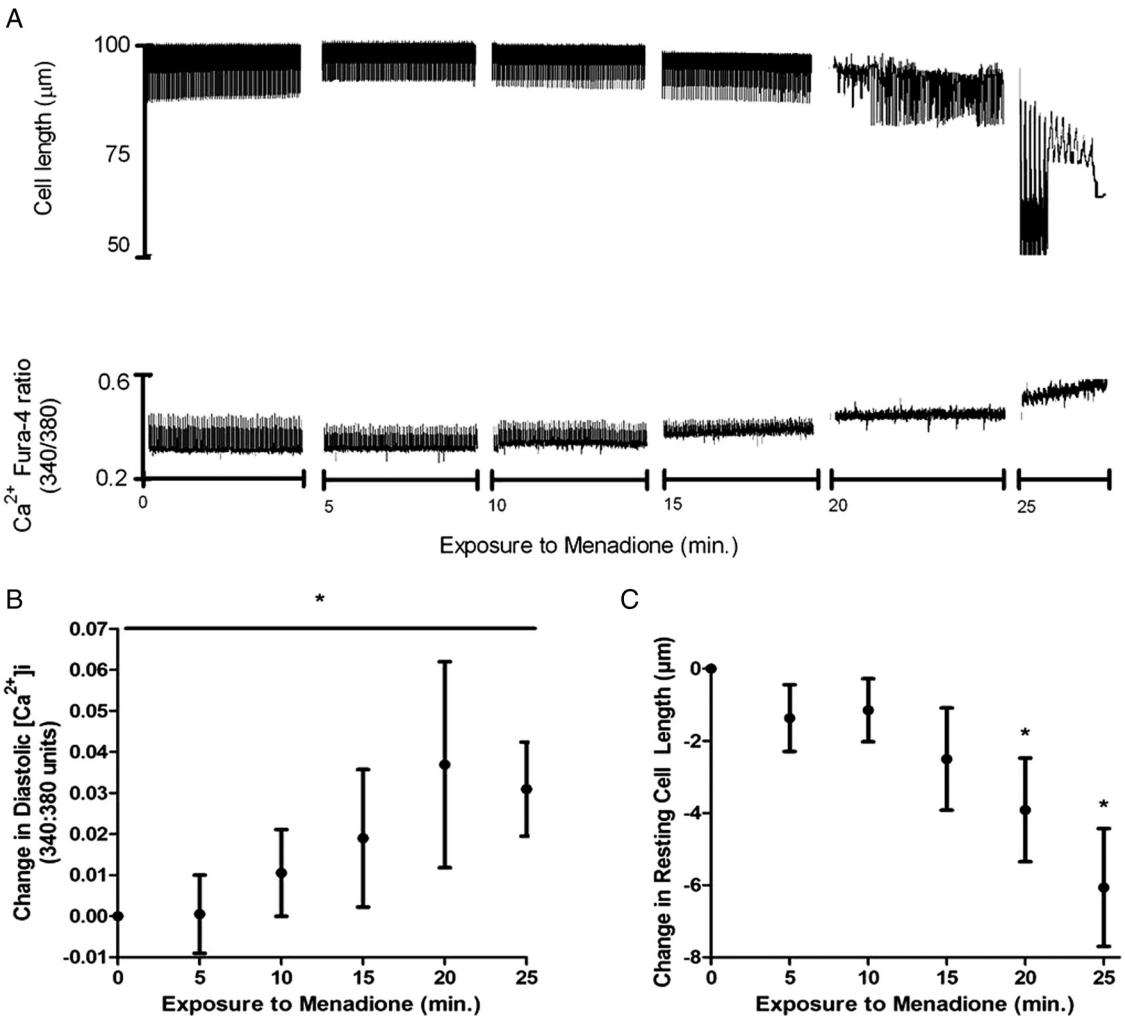

Figure 5. (**A**) Representative recording of cell length (upper trace) and [Ca^{2+}]i (lower trace) in a single myocyte exposed to 50 μM menadione and stimulated at 1 Hz. Traces represent 1 min recordings at 5 min intervals. (**B**) Increase in diastolic [Ca^{2+}]i and (**C**) decrease in resting cell length during exposure to menadione. *$P < 0.05$, 1-way RM ANOVA vs. pre-exposure; $n = 7$ myocytes from $n = 4$ hearts.

myocytes to exhibit early after depolarizations (EADs) or to become unexcitable. Similarly, Matsuura and Shattock (1991) identified an oscillatory transient current that spontaneously triggered SR Ca^{2+} release and automaticity, greatly increasing the risk of dysrhythmias on exposure to rose Bengal (10–100 nM). This current was attributed to the Na^+–Ca^{2+} exchanger and is typically associated with delayed after depolarizations (DADs) (Venetucci *et al.*, 2008). Another novel finding of this study is the demonstration of spontaneous [Ca^{2+}]i transients in intact adult myocytes on exposure to menadione. It can be seen in Fig. 5A that after 20 min exposure to menadione, Ca^{2+} release became irregular and was not coupled to the stimulating pulse, these spontaneous releases of Ca^{2+} are typical of those thought to trigger DAD type arrhythmias. In addition, the dysrhythmia shown in Fig. 4A is consistent with DADs rather than EADs being the arrhythmic mechanism induced by menadione.

ROS production interferes with mitochondrial function. This has been shown to cause a release of Ca^{2+} from the mitochondria (Saxena *et al.*, 1995) and to reduce mitochondrial respiration (Nulton-Persson and Szweda, 2001; Long *et al.*, 2004). Both SERCA, and Na^+ and K^+-ATPase activity are ATP dependent, it is therefore possible that increased [Ca^{2+}]i and ATP depletion potentiate one another; as [Ca^{2+}]i rises so does the cell's utilization of ATP, thus further depleting ATP stores.

A reduction in ATP may trigger the opening of I_{KATP} channels and contribute to the APD shortening that we observed (Brown and O'Rourke, 2010). However, our preliminary studies ($n = 2$) found that 50 μM glibenclamide was not effective at preventing the degree of APD shortening caused by menadione. Oxygen-free radicals have been reported to have a direct effect upon I_{KATP} channels decreasing their sensitivity to ATP (Ichinari *et al.*, 1996; Tokube, Kiyosue and Arita, 1996).

As modification of contractility and electrical activity are interrelated it is useful to measure these parameters simultaneously. A further novel finding is that APD shortening occurs alongside the fall in DP and is likely to contribute to this effect because a shorter APD will lead to less Ca^{2+} influx through L-type Ca^{2+} channels, a smaller SR Ca^{2+} load and thus a smaller SR Ca^{2+} release, in turn smaller SR Ca^{2+} release will generate less inward Na^{+}–Ca^{2+} exchange current, shortening the APD (Bouchard, Clark and Giles, 1995).

Study limitations

Due to the instability of physiological buffer solutions, there is currently no method of exposing cardiac tissue to a known concentration of ROS. Consequently, the concentration of menadione administered was a proxy for the concentration of superoxide anions. Furthermore, although menadione is the most specific superoxide anion donor (Choi *et al.*, 2005), potential effects relating to H_2O_2 and OH^- cannot be excluded.

Conclusion

The data presented provide evidence that exposure to menadione, at 50 μM, exerts a negative inotropic effect as a consequence of an increase in diastolic pressure. Moreover, we have shown that in single LV myocytes this decrease in resting cell length occurs in parallel with an increase in $[Ca^{2+}]i$, which we suggest is a result of the actions of superoxide on Na^{+}–Ca^{2+} exchange and SERCA function.

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Author biography

L.J.R. studied pharmacology in relation to medicine as part of an intercalated degree; he subsequently completed his MBChB at the University of Leeds, passing with honours. He began work as a Foundation Year 1 doctor in August 2013 and is currently considering an academic and research career in Cardiology or Cardiac Surgery. He contributed equally to the conduct of experiments and analysis of data, alongside A.L. and K.W. He also wrote the paper and had primary responsibility for its final content. Aside from the conduct of experiments and analysis of data A.L. and K.W. also edited the paper before final submission. A.L. provided the illustrations. Professor Ed White designed this project as a dissertation for L.J.R., A.L. and K.W.

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