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The K_{ATP} channel opener diazoxide protects cardiac myocytes during metabolic inhibition without causing mitochondrial depolarization or flavoprotein oxidation

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1 The K_{ATP} channel opener diazoxide has been proposed to protect cardiac muscle against ischaemia by opening mitochondrial K_{ATP} channels to depolarize the mitochondrial membrane potential, $\Delta\Psi_m$. We have used the fluorescent dye TMRE to measure $\Delta\Psi_m$ in adult rat freshly isolated cardiac myocytes exposed to diazoxide and metabolic inhibition.

2 Diazoxide, at concentrations that are highly cardioprotective (100 or 200 μM), caused no detectable increase in TMRE fluorescence ($n=27$ cells). However, subsequent application of the protonophore FCCP, which should collapse $\Delta\Psi_m$, led to large increases in TMRE fluorescence ($>300\%$).

3 Metabolic inhibition (MI: 2 mM NaCN + 1 mM iodoacetic acid (IAA) led to an immediate partial depolarization of $\Delta\Psi_m$, followed after a few minutes delay by complete depolarization which was correlated with rigor contracture. Removal of metabolic inhibition led to abrupt mitochondrial repolarization followed in many cells by hypercontracture, indicated by cell rounding and loss of striated appearance.

4 Prior application of diazoxide (100 μM) reduced the number of cells that hypercontracted after metabolic inhibition from $63.7\pm4.7\%$ to $24.2\pm1.8\%$ ($P<0.0001$). 5-hydroxydeanoate (100 μM) reduced the protection of diazoxide ($46.8\pm2.7\%$ cells hypercontracted, $P<0.0001$ *vs* diazoxide alone).

5 Diazoxide caused no detectable change in flavoprotein autofluorescence ($n=26$ cells).

6 Our results suggest that mitochondrial depolarization and flavoprotein oxidation are not inevitable consequences of diazoxide application in intact cardiac myocytes, and that they are also not essential components of the mechanism by which it causes protection.
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Abbreviations: $\Delta\Psi_m$, mitochondrial inner membrane potential; 5-HD, sodium 5-hydroxydecanoate; CN^-, cyanide; DMSO, dimethylsulphoxide; DNP, dinitrophenol; FAD, flavin adenine dinucleotide; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; IAA, iodoacetic acid; MI, metabolic inhibition; TMRE, tetramethylrhodamine ethyl ester

Introduction

Diazoxide is a pharmacological activator of ATP-sensitive K^+ channels (K_{ATP} channels) which has been shown in many studies to exert a protective effect against the damage caused to cardiac muscle by ischaemia followed by reperfusion (reviewed by Gross & Fryer, 1999; Cohen *et al*., 2000). This protective effect mimics that of ischaemic preconditioning seen when a prolonged ischaemic challenge is preceded by a brief period of ischaemia followed by reperfusion. K_{ATP} channels are thought to play a key role in the cardioprotection seen with both K_{ATP} channel openers and ischaemic preconditioning, since in either case protection can be blocked by K_{ATP} channel blockers such as glibenclamide or 5-hydroxydecanoate (Gross & Fryer, 1999; Cohen *et al*., 2000). Protection during ischaemia was initially suggested to result from activation of K_{ATP} channels in the sarcolemma of cardiac muscle, leading to an energy-sparing effect through shortening of the action potential, reduced Ca^{2+} entry and so contractile force (Noma, 1983). However, several studies have shown poor correlation between action potential shortening and protection, and further that protection can occur in the absence of action potentials (Gross & Fryer, 1999). In addition to their expression in the plasma membrane of cells, K_{ATP} channels have been described in the inner membrane of mitochondria (Inoue *et al*., 1991) and attention has recently focussed on these mitoK_{ATP} channels as the proposed mediators of cardioprotection (Garlid *et al*., 1997; Liu *et al*., 1998).

Much of the evidence for the involvement of mitoK_{ATP} channels is pharmacological, based especially on the selectivity of the channel opener diazoxide for mitoK_{ATP} over sarcoK_{ATP} (Garlid *et al*., 1997; Grover & Garlid, 2000). The details of the mechanism by which mitoK_{ATP} channels might cause protection remain obscure, but in

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rabbit myocytes in short-term culture, diazoxide has been reported to increase flavoprotein autofluorescence (Liu *et al*., 1998). This effect was potentiated by either activation of protein kinase C or by adenosine, both manoeuvres known to trigger cardioprotection (Sato *et al*., 1998; 2000). Liu *et al*. (1998) suggested that opening of the mitochondrial K_{ATP} channels might dissipate the inner mitochondrial membrane potential ($\Delta\Psi_m$) established by the protein pump, and that this dissipation accelerates electron transfer by the respiratory chain which if uncompensated by increased production of electron donors, leads to net oxidation of the mitochondria. In isolated cardiac mitochondria, Holmuhamedov *et al*. (1999) have found diazoxide to cause depolarization of $\Delta\Psi_m$ and reduce mitochondrial Ca^{2+} entry, and have suggested that the latter effect might contribute to cardioprotection. Further, they reported that diazoxide (300 μM) could depolarize mitochondria in cultured neonatal rat cardiac myocytes. However, this has not so far been demonstrated in adult myocytes, and Kowaltowski *et al*. (2001), have suggested that the K^+ flux resulting from activation of mitoK_{ATP} would not be sufficient to cause either significant depolarization of $\Delta\Psi_m$ or uncoupling.

We therefore set out to determine whether diazoxide can depolarize $\Delta\Psi_m$ measured more directly in intact freshly isolated adult rat cardiac myocytes using the $\Delta\Psi_m$-sensitive dye tetramethylrhodamine ethyl ester (TMRE). We have been unable to detect any effect of diazoxide on $\Delta\Psi_m$ at concentrations of 100–200 μM, sufficient to cause maximal cardioprotection. In contrast, the protonophore FCCP or metabolic inhibition, which should cause complete depolarization of $\Delta\Psi_m$, increased TMRE fluorescence by >300%. Diazoxide was, however, able to protect cells against the hypercontracture that is normally consequent on metabolic inhibition followed by its removal in isolated cardiac myocytes. We were also unable to detect changes in flavoprotein autofluorescence in response to diazoxide. Our results suggest that mitochondrial depolarization and FAD oxidation are not inevitable consequences of diazoxide application in intact cardiac myocytes, and that they are also not essential components of the mechanism by which it causes cardioprotection.

Methods

Preparation of isolated heart cells

Ventricular myocytes were isolated enzymatically from the hearts of adult male Wistar rats (300–400 g) killed by cervical dislocation. The care and sacrifice of animals conformed to the requirements of the U.K. Animals (Scientific Procedures) Act 1986. The heart was rapidly removed and perfused in the Langendorff mode with collagenase (type I, Sigma) and protease (type XV, Sigma) solution as described previously (Lawrence & Rodrigo, 1999). Cells were then dissociated mechanically and washed twice in normal Tyrode solution (see below). Typically, the isolation procedure gave a 70–90% yield of quiescent, rod-shaped cells. Cells were stored at 10°C in Tyrode with the addition of 5 mM Na pyruvate, and used within 24 h.

Solutions

Isolated ventricular myocytes were allowed to settle on the glass coverslip which formed the base of the 0.5 ml experimental bath and continuously superfused with Tyrode solution containing (in mM): NaCl 135, KCl 6, NaH_2PO_4 0.33, glucose 10, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, titrated to pH 7.4 with NaOH (normal Tyrode). Where used, NaCN (2 mM) and iodoacetic acid (1 mM) were added to Tyrode on the day of the experiment and the pH readjusted to 7.4. Other reagents were added to this solution as described in the text. TMRE was obtained from Molecular Probes and pluronic acid from Sigma.

Drugs

Diazoxide and carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) were from Sigma. Drugs were dissolved in dimethylsulphoxide (DMSO) (Sigma) as stock solutions and diluted into Tyrode. DMSO, at the maximum concentration used of 0.2%, did not have any measurable effect on the parameters studied. Sodium 5-hydroxydecanoate (Sigma) was dissolved directly in Tyrode solution.

Fluorescence imaging for measurement of mitochondrial membrane potential, flavoprotein oxidation and cell length

Cells were visualized with an upright Nikon E600FN microscope using a Nikon 40×, NA 0.8, water-immersion fluor objective lens. Fluorescent dyes were excited with light from a Polychrome II monochromator (T.I.L.L. Photonics), and emitted light was imaged with a PentaMAX cooled CCD camera *via* a Gen IV image intensifier (Princeton Instruments). Image acquisition and analysis were performed with MetaFluor software (Universal Imaging). Mitochondrial membrane potential was measured using the dye tetramethyl rhodamine ethyl ester (TMRE). TMRE is rapidly taken up by live cells and because of its positive charge preferentially distributes into negatively charged cellular compartments. Cells were loaded with 6 μM TMRE in the ester form with the addition of 0.01% pluronic acid for 15 min at room temperature (20–22°C) and then washed for 5 min. TMRE was excited at 475 nm and the fluorescence image was collected above 520 nm after passage through a 505 nm dichroic mirror. To minimise the photodamage that can be caused by mitochondrial membrane potential-sensitive dyes, the excitation light was heavily attenuated with neutral density filters and exposure limited to 25 ms at a frame rate of 0.2 Hz. To record flavoprotein autofluorescence an excitation wavelength of 450 nm was used, neutral density filters were omitted, the exposure time was increased to 50 ms, and emitted light was collected as for TMRE.

Image processing and statistical analysis

Fluorescence images were analysed using MetaFluor software. Fluorescence intensity was measured from a region of interest drawn to include the whole cell. A region of the field without cells was used to measure background fluorescence. After subtraction of background, the relative change in fluorescence was calculated as $\Delta F/F_o$, where F_o is the initial

fluorescence level averaged over 10–20 sample points at the beginning of the recording and ΔF at each sample time t is $(F_t - F_o)$. This procedure sets the baseline fluorescence at zero, while $\Delta F/F_o$ value of 1 corresponds to a doubling in fluorescence intensity. Cell length was measured using MetaMorph software (Universal Imaging). To do this, a threshold of 30% maximum fluorescence was applied to each TMRE fluorescence image to isolate the image of an individual cell, and the length of the cell was then recorded for each frame.

All experiments were carried out at $32 \pm 2^{\circ}C$. Data are presented as the mean ± s.e.mean and the number of cells is shown as n. For statistical analysis, Student's paired or unpaired t-test was used as appropriate.

Results

Diazoxide does not affect mitochondrial membrane potential in rat ventricular myocytes

Since it has been proposed that diazoxide may exert protective effects by depolarizing the mitochondrial inner membrane, we sought to investigate any such depolarization by using the rhodamine dye TMRE, which is sensitive to mitochondrial membrane potential ($\Delta\Psi_m$). We used the cooled CCD camera to collect images of fields that usually contained several ventricular myocytes, and measured fluorescence from separate regions that encompassed the whole of each cell. TMRE is positively charged, and partitions between mitochondria and cytoplasm in a Nernstian fashion. Under the loading conditions we used, TMRE accumulates in mitochondria because of the negative potential of the mitochondrial matrix (about −150 mV relative to the cytoplasm), where it causes autoquenching of fluorescence (Emaus *et al*., 1986; Duchen *et al*., 1998). Depolarization of $\Delta\Psi_m$ leads to a redistribution of TMRE from mitochondria to cytoplasm, with a resulting decrease in quenching and increase in the fluorescence measured from the whole cell. Consistent with this, we measured robust increases in TMRE fluorescence when isolated ventricular cells were exposed to treatments expected to depolarize $\Delta\Psi_m$, such as application of the uncoupler FCCP or metabolic inhibition, as can be seen for example in Figures 1a and 2a.

To investigate possible effects of diazoxide on $\Delta\Psi_m$, TMRE-loaded myocytes were bathed in normal Tyrode before being superfused with diazoxide for 7–10 min. Such an experiment is illustrated in Figure 1a. Diazoxide (200 μM) caused no detectable change in fluorescence, though subsequent exposure of the cell to the protonophore FCCP (5 μM) caused an abrupt increase in fluorescence ($\Delta F/F_o \approx 3$) as expected for depolarization of $\Delta\Psi_m$ (Di Lisa *et al*., 1995; Leyssens *et al*., 1996). We were similarly unable to detect any change in TMRE fluorescence in response to diazoxide in 27 cells to which we applied the drug at either 100 or 200 μM. In each cell we compared the fluorescence averaged over 3 min before the application of diazoxide with that averaged over 3 min in diazoxide (4 min after the initial application of diazoxide). Each cell subsequently gave a large response to either FCCP or metabolic inhibition (MI, see below). Mean results for the effects of diazoxide and FCCP are shown in Figure 1b. There was no significant difference between the fluorescence measured before and during diazoxide in these cells ($P = 0.29$, paired t-test); the mean values of $\Delta F/F_o$ being 0.002 ± 0.003 and 0.021 ± 0.019 ($n = 27$) before and during diazoxide respectively. Thus we have been unable to detect any depolarization of $\Delta\Psi_m$ as indicated by a change in TMRE fluorescence in our experiments.

Changes in $\Delta\Psi_m$ and cell length in response to metabolic inhibition

In single cardiac myocytes, metabolic inhibition (MI), or hypoxia, of sufficient duration, followed by removal of MI or reoxygenation leads to irreversible cell damage indicated by cell shortening to a 'hypercontracted' state with loss of striations and a change from rectangular to rounded cell morphology (Silverman & Stern, 1994). Experiments with the fluorescent dye JC-1, which alters its distribution between monomer and aggregated forms in response to $\Delta\Psi_m$ suggest that MI causes mitochondrial depolarization, and that $\Delta\Psi_m$ can be at least partially restored on restoration of metabolism (Di Lisa *et al*., 1995; Leyssens *et al*., 1996). We therefore used TMRE to measure changes in $\Delta\Psi_m$ in response to MI and its removal and to correlate changes in $\Delta\Psi_m$ with those in cell length. These experiments also allowed us to establish a background procedure for producing hypercontracture against which to assess any protective effects of diazoxide.

Figure 2 shows the effect of inhibition of both oxidative phosphorylation and glycolysis for 10 min with 2 mM NaCN and 1 mM iodoacetic acid ($CN^- + IAA$) on $\Delta\Psi_m$ and cell length. Superfusion with $CN^- + IAA$ caused an immediate partial depolarization of $\Delta\Psi_m$, followed by a further substantial depolarization, reaching a peak in less than 5 min. Depolarization was sustained in the continued presence of $CN^- + IAA$ (Figure 2a). The corresponding changes in cell length and morphology are shown in Figure 2b,c. The onset of the substantial phase of depolarization in $CN^- + IAA$ was followed by abrupt shortening to about 60% of the initial cell length as cells went into rigor contracture, which was fully developed when mitochondrial depolarization reached completion. It is important to note that depolarization occurred prior to cell shortening so that the change in cell length contributed only marginally, if at all, to the increase in fluorescence. Removal of $CN^- + IAA$ lead to rapid repolarization of $\Delta\Psi_m$ to near its initial level. Once repolarization was complete, irreversible hypercontracture occurred, indicated by further cell shortening to around 40% of initial length (Figure 2b) with corresponding changes in morphology (Figure 2d).

The biphasic response of $\Delta\Psi_m$ to combined application of CN^- and IAA suggests that the immediate partial depolarization may result from abolition of electrogenic proton pumping by complex IV by CN^-, with complete depolarization occurring after remaining stores of ATP have been consumed, as suggested by its coincidence with rigor. The effects of separate application of CN^- or IAA shown in Figure 3 are consistent with this explanation. 2 mM CN^- alone caused an immediate sustained partial depolarization (TMRE $\Delta F/F_o = 0.61 \pm 0.12$, $n = 32$, Figures 3a,c), which was rapidly reversible on removal of CN^- and occurred without

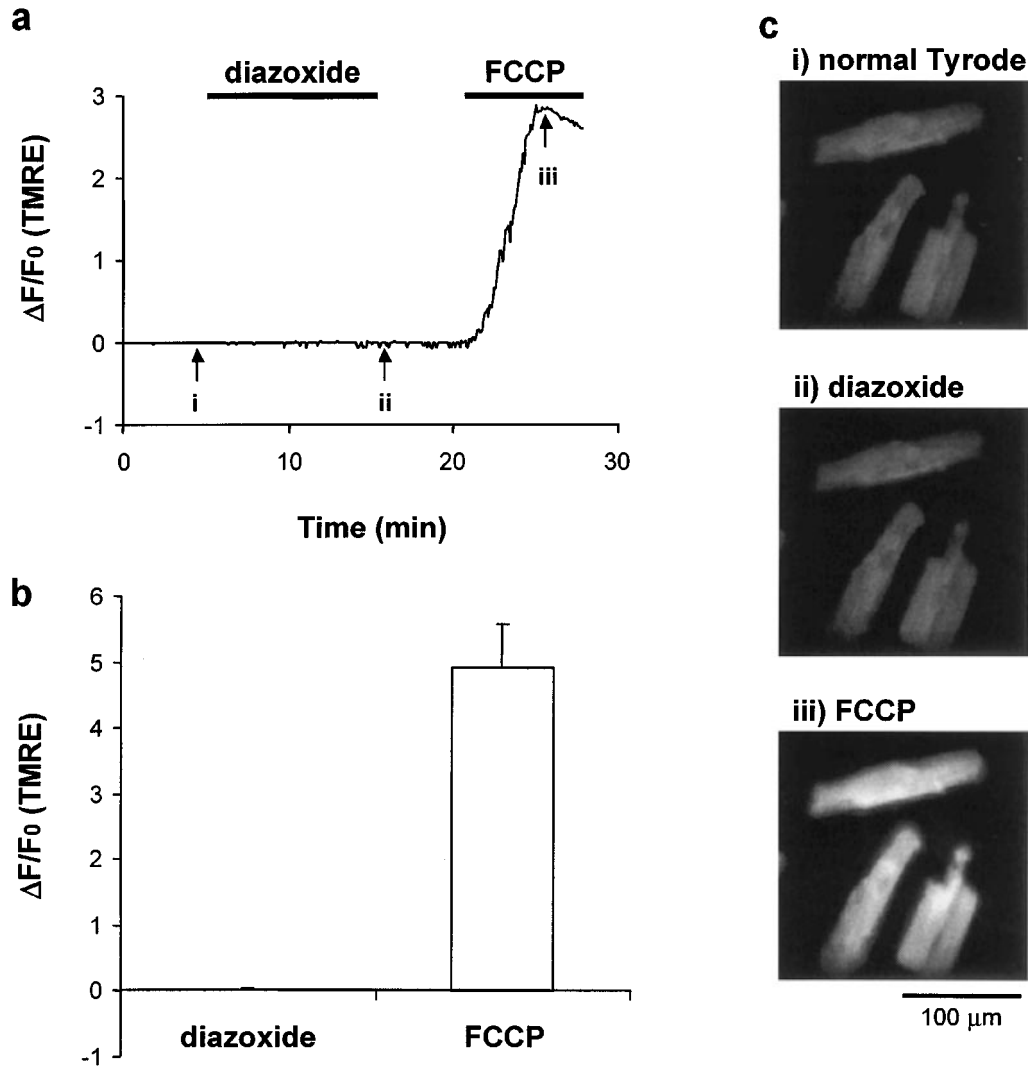

Figure 1 Diazoxide does not alter $\Delta\Psi_m$ indicated by TMRE fluorescence in isolated rat cardiac myocytes. (a) Recording of fluorescence from a single cardiac myocyte loaded with TMRE. Fluorescence was excited at 475 nm for 25 ms at 0.2 Hz, and emitted light at >520 nm was imaged as described in the Methods. In this and subsequent figures, fluorescence was measured for a region of the image that encompassed the whole cell, and is expressed as relative fluorescence $\Delta F/F_o$ (see Methods). Diazoxide (200 μM) and FCCP (5 μM) were applied as indicated. (b) Mean (+s.e.mean) data from 27 cells in experiments like that shown in (a). (c) Images showing a field of four cells before (i), and during (ii) the application of diazoxide and during the response to FCCP (iii). Note the lack of change in fluorescence in response to diazoxide in contrast to the large increase that occurred with FCCP. The timing of the images is indicated on the recording of panel (a).

change in cell length. Subsequent application of FCCP caused an immediate large depolarization of $\Delta\Psi_m$ (Figure 3a). IAA applied alone caused no detectable change in $\Delta\Psi_m$ over 10 min (TMRE $\Delta F/F_o = -0.03 \pm 0.03$, $n=22$, Figure 3b,c), presumably since oxidative phosphorylation was sufficient to maintain $\Delta\Psi_m$ under these conditions. Subsequent addition of CN^- results in changes in TMRE fluorescence essentially the same as those seen when CN^- and IAA are applied together. Mean responses of TMRE fluorescence to CN^- and IAA, either alone or in combination, and to FCCP are shown in Figure 3c. The mean increase in TMRE fluorescence in response to CN^- and IAA was very similar to that caused by FCCP ($\Delta F/F_o = 4.54 \pm 0.36$, $n=48$ and 4.89 ± 0.67, $n=18$, respectively, $P=0.63$), as would be expected if either procedure causes complete mitochondrial depolarization.

Diazoxide protects cardiac muscle cells from hypercontracture after metabolic inhibition

Since we could not detect mitochondrial depolarization in response to diazoxide, we sought to determine whether it could nevertheless protect cells from the damaging effects that follow metabolic inhibition. To do this we used fields of 8–16 cells which were either superfused with Tyrode for 7 min (control) or exposed to 100 μM diazoxide for 5 min followed by 2 min Tyrode. Either group was then exposed to metabolic inhibition with 2 mM $CN^- + 1$ mM IAA for 7 min, after which they were returned to Tyrode with 5 mM pyruvate. Five minutes after this, the number of hypercontracted cells was counted. Figure 4 shows that pretreatment with diazoxide reduced the percentage of cells that went into hypercontracture from $63.7 \pm 4.7\%$ to $24.2 \pm 1.8\%$

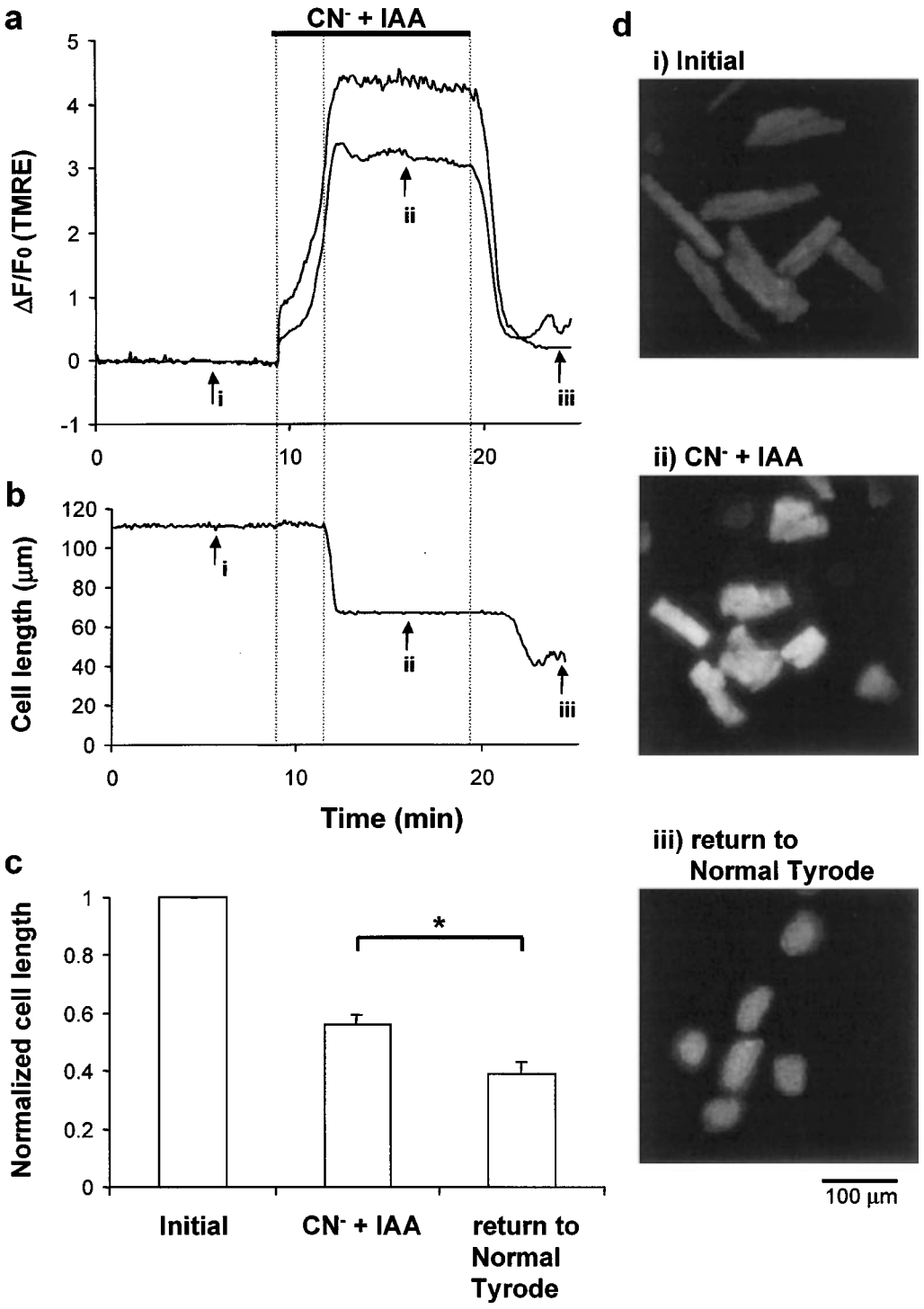

Figure 2 The effect of metabolic inhibition on $\Delta\Psi_m$ and cell length. (a) Recordings of TMRE fluorescence from two cardiac myocytes in response to superfusion with 2 mM NaCN and 1 mM iodoacetic acid ($CN^- + IAA$), applied as indicated. (b) Corresponding changes in cell length, taken from the cell in (a) that gave the smaller fluorescence signal. The shortening to rigor occurs after substantial depolarization of $\Delta\Psi_m$ has occurred, while following removal of metabolic inhibition near complete repolarization precedes shortening into hypercontracture. (c) Mean (+s.e.mean) cell lengths for 17 cells during rigor and after hyercontracture, normalised to the initial length in normal Tyrode for each cell. $^*P<0.001$. (d) Images showing a field of cells before metabolic inhibition (i), in rigor (ii) and in hypercontracture after removal of $CN^- + IAA$ (iii). The timing of the images is indicated on the recordings of panels (a) and (b).

($P<0.0001$). In intact hearts, the protective effects of diazoxide are antagonized by the mitoK_{ATP} channel inhibitor 5-hydroxydecanoate (5-HD), and we therefore tested whether 5-HD could inhibit the protection by diazoxide in isolated myocytes. In these experiments, 5-HD (100 μM) was added with the diazoxide exposure and was present throughout the

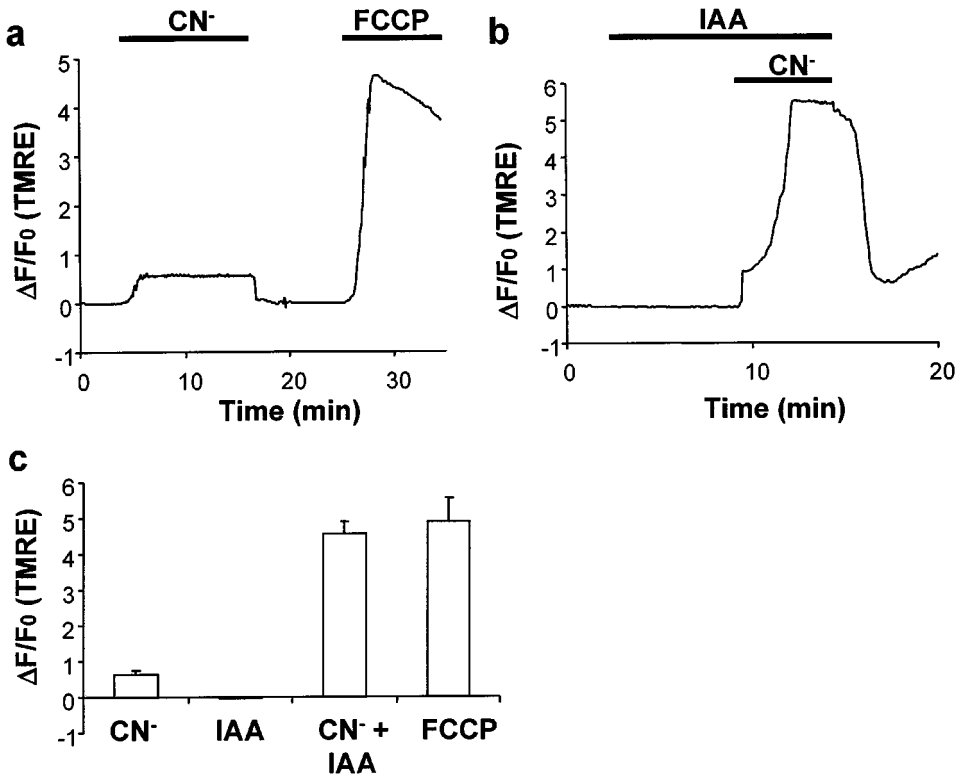

Figure 3 Separating the effects of cyanide and iodoacetic acid on $\Delta\Psi_m$. (a) Recording of TMRE fluorescence in a cardiac myocyte superfused with CN^- (2 mM) and FCCP (5 μM) as indicated. CN^- caused a small and reversible depolarization of $\Delta\Psi_m$, compared to complete depolarization by FCCP. (b) TMRE fluorescence from a myocyte showing the lack of effect of IAA (1 mM) alone, followed by depolarization of $\Delta\Psi_m$ by $CN^- + IAA$. (c) Relative fluorescence in a number of cells exposed to CN^-, IAA, $CN^- + IAA$ and FCCP. Bars show mean (+s.e.mean) values from 32, 22, 48, 18 cells respectively.

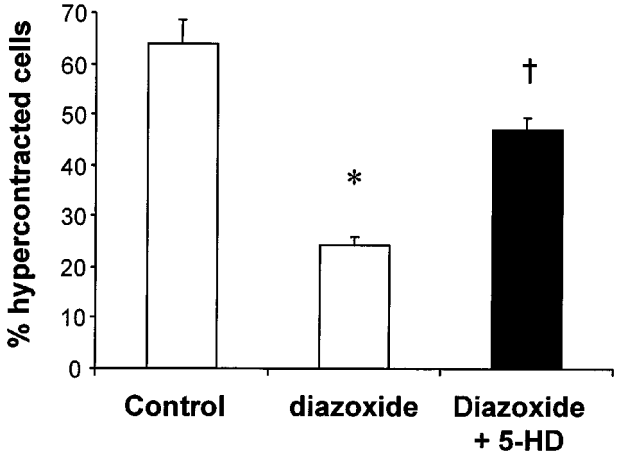

Figure 4 Diazoxide protection against the effects of metabolic inhibition. Mean (+s.e.mean) data showing the effect of diazoxide pretreatment (100 μM) and diazoxide in the presence of 5-hydroxydecanoate (100 μM) on the percentage of hypercontracted cells 5 min after removal of metabolic inhibition. Results are from 13, 15 and eight experiments (138, 150 and 62 cells) in control, diazoxide, and diazoxide + 5-HD respectively. $^*P<0.0001$ compared to control. $\dagger P<0.0001$ compared to diazoxide alone.

subsequent metabolic inhibition and its removal. 5-HD reduced the protective effect of diazoxide, increasing the percentage of hypercontracted cells to $46.8 \pm 2.7\%$ ($P<0.0001$ *vs* diazoxide alone).

The effect of diazoxide on FAD autofluorescence

In rabbit ventricular myocytes in short-term culture, diazoxide causes an increase in flavoprotein autofluorescence (Liu *et al.*, 1998). Since we have been unable to detect mitochondrial depolarization in freshly isolated rat cardiac myocytes, we have looked for increases in flavoprotein autofluorescence in response to diazoxide in these cells. Figure 5a shows an example of such an experiment. Application of diazoxide (100 μM) had no effect on flavoprotein autofluorescence, which however fell in response to $CN^- + IAA$, and increased with dinitrophenol (DNP, 200 μM) as expected if these caused flavoprotein reduction and oxidation respectively. The mean results for a number of cells are shown in Figure 5b, confirming that diazoxide (100 μM, 10 min) did not affect flavoprotein autofluorescence ($\Delta F/F_o = 0.034 \pm 0.03$; $n=26$), while $CN^- + IAA$ and DNP caused a decrease and increase respectively to -0.67 ± 0.06 ($n=32$) and 2.09 ± 0.23 ($n=23$), respectively. We conclude that diazoxide, at concentrations that are protective against the effects of metabolic inhibition, did not cause substantial flavoprotein oxidation in rat ventricular myocytes.

Discussion

The results that we present in this paper show that, in ventricular myocytes freshly isolated from rat heart, diazoxide can give protection against the damage consequent

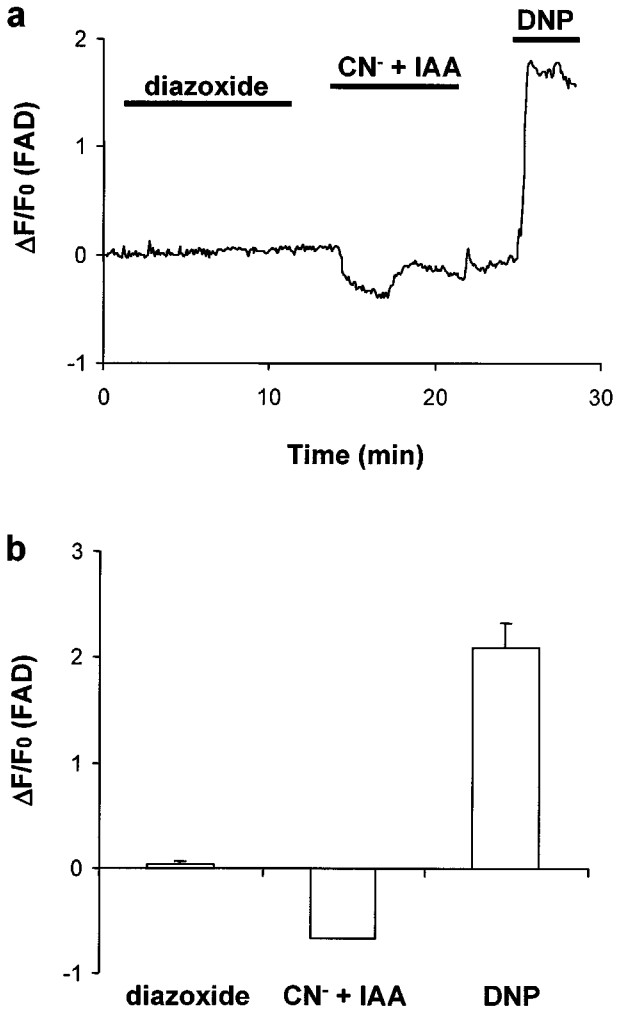

Figure 5 Diazoxide and flavoprotein autofluorescence. (a) Recording of flavoprotein (FAD) autofluorescence from a cardiac myocyte. Excitation was at 450 nm for 50 ms at 0.2 Hz, emitted light at >520 nm was imaged. Diazoxide (100 μM), CN^- + IAA and dinitrophenol (DNP, 200 μM) were added as indicated. (b) Mean (+s.e.mean) data from 26, 32 and 23 cells exposed to diazoxide as in (a) and/or to CN^- + IAA or DNP.

upon simulated ischaemia and reperfusion. Such a protective effect is in agreement with the reported effect of diazoxide in protecting intact rat hearts subjected to ischaemia and reperfusion as measured by improved recovery of function and reduced infarct size (Garlid *et al*., 1997; Fryer *et al*., 2000). However, under the same conditions, we were unable to detect any change in mitochondrial membrane potential, $\Delta\Psi_m$, estimated using the dye TMRE. In contrast, application of the protonophore FCCP or inhibition of both glycolysis and oxidative phosphorylation both increased TMRE fluorescence in a manner consistent with their causing complete depolarization of $\Delta\Psi_m$, as has been reported previously (Di Lisa *et al*., 1995).

Since we detected no change in $\Delta\Psi_m$ in response to diazoxide in any cells studied, it is interesting to estimate how small a change we might have expected to resolve in our experiments. It is important to note that in each of these cells we subsequently observed a large increase in TMRE fluorescence, usually of 400–500% ($\Delta F/F_o = 3-4$), in response to either FCCP or metabolic inhibition (e.g. Figures 1a, 2a and 3c). To estimate the detectable change in fluorescence we calculated the standard error of our measurement of initial fluorescence in the absence of any metabolic or other challenge as $\Delta F/F_o = 0.004$. A measurement $3 \times$ s.e.mean from the mean fluorescence would occur with $P < 0.01$, and taking this as the minimum detectable change in fluorescence gives an estimate for this change as $\Delta F/F_o = 0.012$. To interpret this in terms of depolarization we need to know the relation between TMRE fluorescence and $\Delta\Psi_m$. This has not been determined in intact cells, but in suspensions of rat cardiac mitochondria, Emaus *et al*. (1986) found that the fluorescence resulting from dequenching of rhodamine 123 on mitochondrial depolarization was linearly related to $\Delta\Psi_m$. If we assume such a linear calibration for TMRE, which dequences in an essentially similar way (Duchen *et al*., 1998), and that the fluorescence increase seen with FCCP corresponds to a maximal depolarization of 150 mV, our minimum detectable change in fluorescence would correspond to a change in $\Delta\Psi_m$ of <1 mV.

Our finding that diazoxide does not cause depolarization of $\Delta\Psi_m$ in intact myocytes agrees with the proposal of Garlid (2000), who has estimated that K^+ flux through mitoK_{ATP} channels would be insufficient to cause significant depolarization. In isolated mitochondrial preparations, diazoxide and other K_{ATP} channel openers have been reported to depolarize $\Delta\Psi_m$ to some extent (Holmuhamedov *et al*., 1999). However, Kowaltowski *et al*. (2001) have argued that these studies in isolated mitochondria represent effects that are independent of mitoK_{ATP} since the conditions (absence of Mg^{2+} and ATP) were such that mitoK_{ATP} channels should already be open. Further studies of the conditions under which mitochondrial depolarization does or does not occur in intact myocytes or even intact cardiac tissue will be of great interest.

We also did not detect flavoprotein oxidation, as indicated by increased autofluorescence, in response to diazoxide (Figure 5), although in the same cells CN^- or dinitrophenol caused the expected reduction and oxidation respectively. This contrasts with the findings of Liu *et al*. (1998) and Sato *et al*. (2000) using rabbit myocytes, who have argued that oxidation results from an uncoupling effect of mitoK_{ATP} opening. It is possible that our contrasting findings reflect differences in the metabolic state of the cells and in experimental conditions. We used freshly isolated myocytes in physiological saline, and worked at 32°C, whereas the experiments on rabbit myocytes used cells kept in culture medium for up to 2 days, and measured fluorescence in glucose free solution at room temperature (Liu *et al*., 1998; Sato *et al*., 2000).

Our experiments suggest that net flavoprotein oxidation (and mitochondrial depolarization) are not essential steps in protection by diazoxide, since we observed protection without flavoprotein oxidation. It is possible that an uncoupling effect of diazoxide through mitoK_{ATP} opening occurred in our experiments, but did not result in net flavoprotein oxidation because of the availability of glucose as a substrate. Alternatively, Kowaltowski *et al*. (2001) have suggested that protection by diazoxide is unrelated to flavoprotein oxidation, but rather that the protective action of diazoxide against ischaemia occurs *via* a different mechanism; opening of mitoK_{ATP} channels leading to preservation of mitochondrial

matrix volume and so of the integrity of enzymes situated between the outer and inner mitochondrial membranes. Our results are consistent with this suggestion and also with results published recently by Ovide-Bordeaux *et al*. (2000) who used intact permeabilized rat cardiac muscle fibres and found that diazoxide did not affect mitochondrial respiration except when succinate was used as a substrate. In rabbit myocytes, measurement of flavoprotein fluorescence has provided attractive evidence for the hypothesis that mito-K_{ATP} channel activation underlies cardioprotection by pharmacological or ischaemic preconditioning (Liu *et al*., 1998; Sato *et al*., 1998; 2000). Our experiments suggest that flavoprotein fluorescence can not always be used as a measure of mitoK_{ATP} activation, and in this context future studies that define the exact relationship between mitoK_{ATP} channel opening and flavoprotein fluorescence under different conditions will be important. Two key elements which would greatly strengthen the hypothesis that mitoK_{ATP} channels lay a key role in cardioprotection are a clearly defined mechanism by which they might induce protection and identification of the molecular structure of the channel itself.

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