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The effect of oxygen free radicals on calcium current and dihydropyridine binding sites in guinea-pig ventricular myocytes

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1 We used electrophysiological and binding techniques to determine the effects of oxygen free radicals (OFRs) generated by dihydroxyfumaric acid (DHF, 5 mM) on calcium current and dihydropyridine binding sites in guinea-pig isolated ventricular myocytes.

2 Binding of [^{3}H]-PN200–110 to isolated ventricular myocytes revealed one population of binding sites with a K_D of 0.11 ± 0.01 nM and B_{max} of 139.1 ± 6.9 fmol mg^{-1} protein ($n=24$). After 15 min of exposure to DHF, the density, but not the affinity of [^{3}H]-PN200–110 binding sites was significantly ($P<0.01$) reduced to 35% of the control value ($B_{max}=49.4 \pm 3.7$ fmol mg^{-1} protein, $K_D=0.11 \pm 0.01$ nM, $n=15$). In the presence of superoxide dismutase (SOD) and catalase (CAT) the reduction in [^{3}H]-PN200–110 binding sites was almost completely prevented ($B_{max}=120.5 \pm 7.4$ in control, $n=4$ and 98.8 ± 7.4 fmol mg^{-1} protein in DHF plus SOD and CAT, $n=4$). K_D values were not modified (0.08 ± 0.01 in control and 0.09 ± 0.01 nM in DHF plus SOD and CAT).

3 The time-course of the reduction of [^{3}H]-PN200–110 binding sites by OFRs was paralleled by the decrease in L-type calcium current ($I_{Ca,L}$) measured in patch-clamped guinea-pig ventricular myocytes either in the absence or in the presence of EGTA in the patch pipette. In the former conditions OFRs induced the appearance of calcium-dependent alterations, i.e. the transient inward current, within 10 min. After 30 min of incubation with DHF, [^{3}H]-PN200–110 binding sites were reduced to 25% of the control value.

4 In myocytes incubated with the antilipoperoxidant agent, butylated hydroxytoluene (BHT, 50 μM), the decrease in [^{3}H]-PN200–110 binding sites caused by DHF was partially prevented (B_{max} values after 30 min exposure to DHF were 55.5 ± 1.9 and 23.7 ± 5.9 fmol mg^{-1} protein in the presence and in the absence of BHT respectively, $P<0.05$). BHT did not affect the decrease in [^{3}H]-PN200–110 binding sites during the first 15 min of exposure to DHF, but was able to prevent completely the further decrease occurring during the following 15 min of incubation with OFRs.

5 Our results demonstrate that the OFR-induced decrease in calcium current is associated with a reduction in DHP binding sites. The decrease in calcium current and in calcium channels may be implicated in the mechanical dysfunction associated with oxidative stress.

Keywords: Oxygen free radicals; isolated myocytes; L-type calcium current; dihydropyridine receptor binding; butylated hydroxytoluene

Introduction

Oxygen-derived free radicals (OFRs) are thought to be implicated in the deleterious events which are associated with the reperfusion of the ischaemic myocardium, such as stunning and arrhythmias (Burton *et al.*, 1984; Bolli, 1988; McCord, 1988; Hearse, 1990).

Several studies have clearly documented that OFRs may cause cellular electrophysiological alterations which are considered to be arrhythmogenic: exogenous sources of OFRs applied either intracellularly (Jabr & Cole, 1993) or extracellularly (Barrington *et al.*, 1988; Beresewicz & Horachova, 1991; Cerbai *et al.*, 1991; Mugelli *et al.*, 1995) may cause action potential prolongation, loss of excitability, and development of both early- and delayed afterdepolarizations. We and other groups have shown in guinea-pig and rat isolated myocytes that these changes are the consequence of intracellular calcium loading (Beresewicz & Horackova, 1991; Cerbai *et al.*, 1991; Jabr & Cole, 1993; Mugelli *et al.*, 1995), possibly involving abnormalities in sarcoplasmic reticulum Ca^{2+} release (Shattock *et al.*, 1990; Boraso & Williams, 1994). In fact, prolongation of action potential duration and development of early- (EAD) and delayed afterdepolarizations (DAD) are prevented by intracellular EGTA (Cerbai *et al.*, 1991; Jabr & Cole, 1993; Mugelli *et al.*, 1995) and/or by pretreatment of the cells with ryanodine (Beresewics & Horachova, 1991).

One of the main pathways for calcium entry into the cardiomyocyte is through L-type calcium channels. The effects of OFRs on L-type calcium current ($I_{Ca,L}$) are not consistent but the majority of the studies describe a decrease in the peak current when myocytes are exposed to an external source of OFRs (Shattock *et al.*, 1990; Cerbai *et al.*, 1991; Tarr & Valenzeno, 1991; Nakaya *et al.*, 1992; Coetzee & Opie, 1992; Gill *et al.*, 1995; Mugelli *et al.*, 1995). The decrease in the current could be due to a direct interaction of OFRs with the L-type Ca-channels or to a Ca-dependent inactivation as a consequence of the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) caused by OFRs through different pathways (Kaneko *et al.*, 1990; Shattock *et al.*, 1990; Holmberg *et al.*, 1991; Shattock & Matsuura, 1993).

A direct relationship between dihydropyridine (DHP) binding sites and function of L-type calcium channels has been demonstrated (Lew *et al.*, 1991) and modifications in number and affinity of DHP binding sites have been used as an index of the functional status of L-type calcium channels (Stanfield, 1986; Kaneko *et al.*, 1991). A decrease in DHP binding sites has been consistently obtained in cardiac membranes exposed to oxidant stress (Kaneko *et al.*, 1989b; Matucci *et al.*, 1992). However, membranes prepared from the whole heart derive from different cell types which, as the vascular cells, may contain DHP binding sites, and which may be affected differ-

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ently by the oxidative stress. This condition obviously makes any attempt to relate the decrease in DHP binding sites with any functional effect, such as a decrease in $I_{Ca,L}$ impossible. Since to date the effects of OFRs on DHP binding sites on isolated cardiac myocytes are not known, we decided to evaluate the changes in DHP binding parameters (density and affinity) in guinea-pig ventricular myocytes, by use of the same source of OFRs that we previously demonstrated reduced $I_{Ca,L}$ in these cells (Cerbai *et al.*, 1991). Furthermore, we tried to correlate the time course of the effects of OFRs on DHP binding sites with those on calcium current. We reasoned that this could give some insight into the mechanisms by which OFRs decrease $I_{Ca,L}$ i.e. through a direct or an indirect interaction with the calcium channels, and into the relationship between binding sites and channel function.

Methods

Preparation of ventricular myocytes

The investigation conforms to the rules for the care and use of laboratory animals of the European Community (86/609/CEE). Myocytes were isolated by using a protocol based on previously described procedures for rat hearts (Cerbai *et al.*, 1995). Briefly, hearts were removed from guinea-pigs weighing 200–300 g, and perfused in a Langendorff apparatus at 35°C for 10 min with a nominally zero calcium solution (LCS) of the following composition (mM): NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 20, taurine 20, HEPES, and pyruvic acid 5, gassed with 100% O_2, pH 7.2.

After perfusion with LCS, the perfusion was switched to the same buffer containing 0.1% collagenase, 0.1% albumin and 0.01% dispase. The perfusion was terminated when the tissue became soft (after 10–15 min). The heart was then removed and the ventricles were chopped into small pieces and slowly stirred in LCS to allow cells to be dispersed and collected; the preparations used for the experiments contained 60–80% of rod-shaped cells. For each binding experiment, isolated ventricular myocytes obtained from the same hearts were divided into aliquots and used for different, parallel, experimental procedures.

[^{3}H]-PN200–110 binding assay

Saturation binding assays for dihydropyridine receptors were performed in duplicate by using samples of 5×10^4 myocytes for each assay. Proteins were determined by the method of Lowry (1951) at the end of the experiments. Myocytes, suspended in LCS, were incubated with increasing concentrations (0.01–1.4 nM) of the dihydropyridine calcium antagonist [^{3}H]-PN200–110 in a final volume of 3 ml. Incubation time was 20 min at 37°C. The non-specific binding was determined in the presence of 10 μM nifedipine. The binding assay was carried out in a darkened room under a sodium lamp to prevent the degradation of [^{3}H]-PN200–110 and nifedipine. The reaction was stopped by rapid vacuum filtration over Whatman GF/C glass fibre filters, using a Brandel cell harvester. Filters were washed twice with 5 ml of 50 mM Tris HCl (pH 7.4) at 4°C, then were placed in liquid scintillation vials, containing 5 ml of Aquassure. Radioactivity retained on filters was determined using a Beckman Liquid Scintillator Spectrometer at 55% efficiency.

Oxygen free radical-generating solutions

The oxygen free radical (OFR)-generating solution was prepared just before use and consisted of 5 mM dihydroxyfumarate (DHF) added to the LCS and adjusted to pH 7.35 with NaOH. The solution was bubbled with 100% O_2. To stop exposure to oxygen radicals, myocytes were separated from DHF by centrifugation at $350 \times g$ for 1 min. The pellet was immediately resuspended in 10 ml of LCS and then diluted to a final concentration of 25×10^4 cells ml^{-1}.

In the experiments designed to determine the effect of superoxide dismutase (SOD) and catalase (CAT, the scavenging enzymes were used at a final concentration of 600 u ml^{-1} and 2500 u ml^{-1} respectively.

Butylated hydroxytoluene (BHT, 50 μM), an antilipoperoxidant agent, was used to prevent the effect of formation of lipid hydroperoxide. Aliquots of myocytes were incubated in BHT just after isolation, and maintained in BHT-containing medium (either in control or in OFR-generating solutions) throughout the experiment.

Measurement of calcium current

The experimental set-up was similar to that described in Cerbai *et al.* (1995). A drop containing cells was placed in the experimental chamber (0.2 ml) and superfused by means of a peristaltic pump (Masterflex, model 7524/05, Cole-Parmer Instrument Company) at a flow rate of 1.8 ml min^{-1}; a three-line system controlled by electronic valves allowed solutions to be changed rapidly. The recording chamber was mounted on the stage of an inverted microscope (TMS, Nikon). The control solution was a modified Tyrode's solution containing (in mM: NaCl 137, KCl 5.4, $CaCl_2$ 5.4, $MgCl_2$ 1.2, HEPES 5, glucose 10; pH was adjusted to 7.35 with NaOH, the temperature was kept at 36±0.5°C. The oxygen-radical-generating solution consisted of 5 mM DHF added to the Tyrode solution and adjusted to pH 7.35 with NaOH. The solutions were bubbled with 100% O_2. The internal solution of the patch pipettes contained (in mM): KCl 140, $MgCl_2$ 1, Na_2-ATP 5, ethyleneglycol-bis (β-aminoethyl ether)-*N,N,N'N'*-tetra acetic acid (EGTA) 1, HEPES 10, adjusted to pH 7.20 with KOH. The intracellular calcium activity ($[Ca^{2+}]_i$) was estimated to be less than 50 nM; EGTA was not included in the pipette solution in some experiments, as indicated when appropriate.

The whole-cell configuration of the patch-clamp technique was used. The electrical signal was recorded by a patch amplifier (Axopatch 1D, Axon Instrument Inc.), digitised (Labmaster TL-1 DMA, Scientific Solutions), and displayed on the monitor of a 386 personal computer and a digital oscilloscope (Nicolet 310, Nicolet Instrumentation Company). The cut-off frequency was 20 kHz. Voltage protocol generation, data acquisition, and analysis were performed with the pClamp software (Vers 5.5.1, Axon Instrument Inc.). MicroCal Origin (MicroCal Software Inc.) was used for further analysis.

Recording was started after 5 min of dialysis of the cell, in order to allow stabilization of membrane currents. The voltage protocol, applied at a rate of 0.1 Hz, consisted of three steps: from a holding potential of −80 mV, cells were depolarized to +40 mV for 500–700 ms to evoke time-dependent K currents (I_K) and then kept at −40 mV for another 1.5 s to measure tail currents of I_K. These currents were sampled at 1 kHz. Then, a 160-ms step to 0 mV was applied to activate $I_{Ca,L}$ which was sampled at 5 kHz. $I_{Ca,L}$ amplitude was measured as difference between steady state current, measured at the end of the depolarizing step at 0 mV, and peak inward current.

Materials

The following substances were used: (+)-[methyl-^{3}H]-PN200–110 ((+)-4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid methyl isopropyl ester), specific activity 87 Ci mmol^{-1} (Amersham, Buckinghamshire, U.K.); Aquassure (NEN Research Products, Boston, MA, U.S.A.); collagenase (Type I), dihydroxyfumaric acid (DHF), butylated hydroxytoluene (BHT), HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid), nifedipine and superoxide dismutase (SOD) (Sigma Chemical Co., St. Louis, MO, U.S.A.); dispase, albumin (fatty acid free fraction V), and catalase (CAT) (Boehringer, Mannheim, F.R.G.).

All other reagents were of analytical grade and obtained from commercial sources.

Data analysis

Saturation experiments were analysed by using the LIGAND Computer program (Munson & Rodbard, 1980). Comparison between two groups was performed by means of Student's *t* test for grouped data; a *P* value of less than 0.05 was considered significant.

Results

Effect of OFR on DHP-binding sites

Binding of [^{3}H]-PN200–110 to isolated ventricular myocytes revealed one population of binding sites with a K_D of 0.11 ± 0.01 nM and B_{max} of 139.1 ± 6.9 fmol mg^{-1} protein ($n=24$). Figure 1 shows that in isolated myocytes incubated for 15 min with OFRs generated by DHF, the density, but not the affinity of [^{3}H]-PN200–110 binding sites was significantly reduced to 35% of the control value: B_{max} was in fact, 49.4 ± 3.7 fmol mg^{-1} protein ($P<0.01$ vs. control) while the affinity was completely unchanged ($K_D = 0.11 \pm 0.01$ nM, $n=15$). To prove that the effect caused by incubation with DHF was actually due to the generation of OFRs, we evaluated the K_D and B_{max} values of [^{3}H]-PN200–110 binding in myocytes incubated in the OFR-generating solution (DHF) plus the scavenging enzymes superoxide dismutase (SOD) and catalase (CAT). The presence of SOD and CAT was able to prevent almost completely the reduction in [^{3}H]-PN200–110 binding sites caused by DHF. B_{max} values were 120.5 ± 7.4 in control ($n=4$) and 98.8 ± 7.4 fmol mg^{-1} protein in DHF plus SOD and CAT ($n=4$). K_D values were not modified, being 0.08 ± 0.01 in control and 0.09 ± 0.01 nM in DHF plus SOD and CAT.

Time course of OFR effect on calcium current and DHP-binding sites

Figure 2 shows the time-course of the reduction in the density of [^{3}H]-PN200–110 binding sites caused by exposure to OFRs, compared with the decrease in calcium current measured in two different experimental conditions (presence or absence of EGTA in the patch pipette). Points at 2 and 5 min with 200 μM or without EGTA have been derived from previously published (Cerbai *et al.*, 1991) results. This analysis was done to try to correlate the change in the density of the DHP binding sites with the development of the effects of OFRs on calcium current, that, as we have previously demonstrated (Cerbai *et al.*, 1991), was reduced by OFRs. A decrease in B_{max} developed over time during exposure to OFRs. After 30 min incubation with DHF, [^{3}H]-PN200–110 binding sites were reduced to 25% of the control value. The greatest part of the decrease developed during the first 15 min (up to 35% of the control value) indicating that two different mechanisms might be involved in the phenomenon.

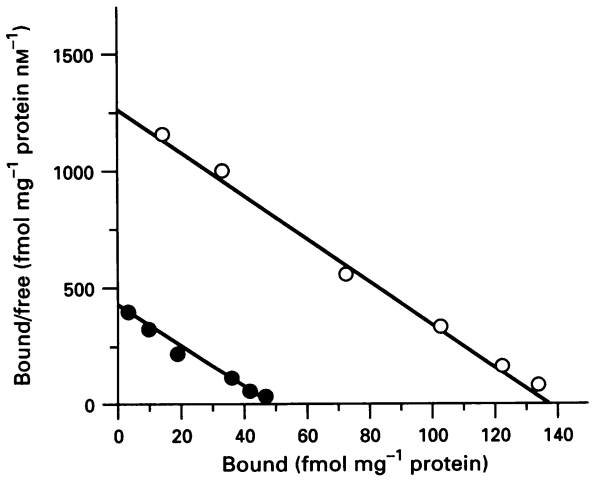

Figure 1 Scatchard plot of [^{3}H]-PN200–110 binding in control myocytes (○) and in myocytes exposed to the oxygen radical-generating solution (DHF) (●). Curves are representative of 24 experiments for the control and 15 experiments for the DHF-exposed myocytes; each experiment was performed in duplicate. Myocytes were incubated for 15 min in oxygenated (100% O_2) zero calcium solution (LCS) at 37°C in the absence or presence of 5 mM DHF and then centrifuged and resuspended in LCS prior to the assay for [^{3}H]-PN200–110 binding.

Effect of OFR on calcium current

The decrease in DHP binding sites in the first 15 min, was paralleled by the decrease in calcium current measured in patch clamped guinea-pig ventricular myocytes either in the absence or in the presence of EGTA in the patch pipette. Representative recordings from which some of the points shown in Figure 2 were taken, are shown in Figure 3. The two panels show the time course of the effects of DHF on the currents recorded in ventricular myocytes, applying the protocol shown in the bottom right corner. Tail currents (representing I_K deactivation) were recorded upon return to −40 mV. From this potential, at which Na channels are inactivated, calcium current was evoked. It is apparent that DHF caused a time-dependent reduction in calcium current clearly visible in the right parts of the panels, where the traces are shown at a fast time scale, and a time-dependent decrease in the outward currents (left side of the panels at a slow time scale). Since the recording pipette did not contain EGTA (panel a), rate of inactivation of the calcium current was fastest (due to a higher $[Ca^{2+}]_i$) compared to the recordings shown in panel (b). Under this experimental situation, 10 min exposure to DHF caused the appearance of the transient inward current (panel a, indicated by the bottom arrow). Consequently, under these experimental conditions it was not possible to follow for a longer period of time the effects of DHF on calcium current. With EGTA in the pipette, (panel b) the manifestations of calcium overload were

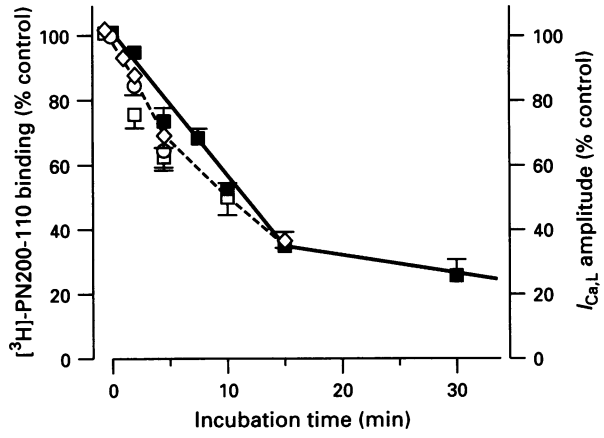

Figure 2 Time-course of the effect of oxygen free radicals (OFR) on number of dihydropyridine binding sites ([^{3}H]-PN200–110 binding, ■) and peak amplitude of L type calcium current ($I_{Ca,L}$, open symbols) in isolated ventricular myocytes. In the receptor binding experiments, cells were preincubated in the OFR-generating solution at 37°C for the indicated times, before centrifugation and resuspension in zero calcium solution (LCS) for [^{3}H]-PN200–110 binding assay. Each value is the mean and s.e.mean of 3 to 5 different experiments performed in duplicate. Peak $I_{Ca,L}$ was measured in patch-clamped cells before and during superfusion with oxygenated Tyrode solution containing 5 mM dihydroxyfumaric acid using a patch pipette solution with 200 μM (○) or 1 mM EGTA (◇) or without EGTA (□). Each point is the mean and s.e.mean of 2 to 6 different measurements; points at 2 and 5 min with 200 μM (○) or without EGTA (□) in the pipette solution were taken from Cerbai *et al.* (1991).

avoided and the effects of DHF were followed for a longer time, during which reduction of calcium current progressed even more. By comparing the recordings shown in (a) and (b) (right), it is apparent, however, that OFRs caused a similar time-dependent reduction of calcium current amplitude in both experimental conditions.

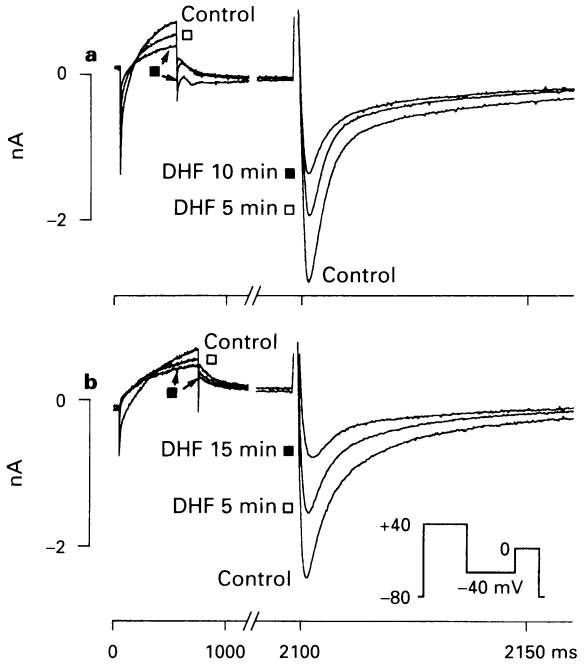

Figure 3 Effect of oxygen free radicals (OFRs) on membrane currents recorded from patch-clamped myocytes, in the absence (a) or presence (b) of 1 mM EGTA in the pipette solution. Each panel shows superimposed current tracings evoked by the voltage protocol shown in the bottom right corner, in control and after 5-, 10- or 15-min exposure to 5 mM dihydroxyfumaric acid (DHF). Note that outward potassium currents (left side of each panel) are shown at a slower time scale than $I_{Ca,L}$ (right side). See text for details.

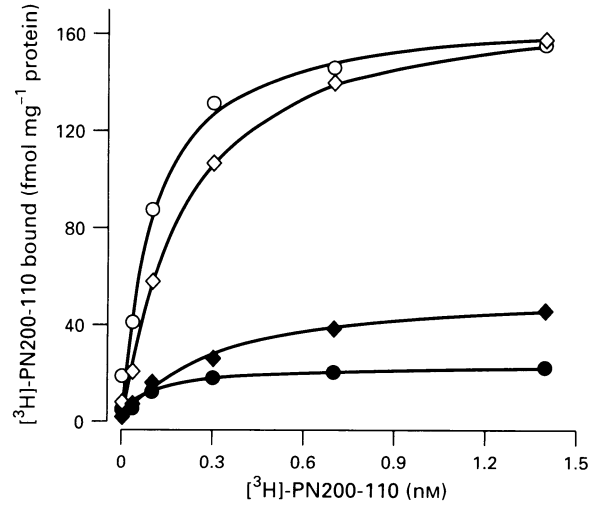

Figure 4 Effect of the antilipoperoxidant agent butylated hydroxytoluene (BHT, 50 μM) on oxygen free radical (OFR)-induced decrease of [^{3}H]-PN200–110 binding. Saturation binding assays of [^{3}H]-PN200–110 were performed in isolated cardiomyocytes pre-incubated (1 h) in zero calcium solution (LCS) in the absence (control, ○) or in the presence of BHT (◇) and exposed for 30 min to a normally oxygenated solution or to the OFR-generating solution (● = dihydroxyfumaric acid (DHF); ◆ = DHF and BHT).

Effect of BHT on OFR-induced Reduction of DHP Binding Sites

Since it is known that production of OFRs promotes peroxidation of membrane lipids (Mak *et al.*, 1983; Ambrosio *et al.*, 1991; Sakomoto *et al.*, 1991), the effect of BHT, a well characterized antioxidant agent (Mikuni *et al.*, 1987), on OFR-induced reduction of DHP binding sites, was evaluated. A typical experiment is shown in Figure 4: [^{3}H]-PN200–110 binding was evaluated in myocytes isolated from the same heart and incubated with or without DHF for 30 min in the absence or in the presence of BHT. As expected, BHT *per se* did not alter the number of [^{3}H]-PN200–110 binding sites (see also Figure 5a). A decrease in affinity was observed: the K_D value was increased from 0.11 to 0.36 nM. As shown in Figure 5b, this effect on K_D was also maintained in the presence of DHF, and it is possibly the consequence of the high lipophilicity of the compound. However, the most important finding was that BHT partially prevented the decrease in [^{3}H]-PN200–110 binding sites caused by 30 min exposure to DHF (Figure 4, B_{max} values were 55.5 ± 1.9 and 23.7 ± 5.9 fmol mg^{-1} protein in the presence and in the absence of BHT respectively, $P < 0.05$). It is worth noting that BHT did not affect the decrease in [^{3}H]-PN200–110 binding sites developing within the first 15 min of exposure to DHF (B_{max} values were 52.3 ± 5.5

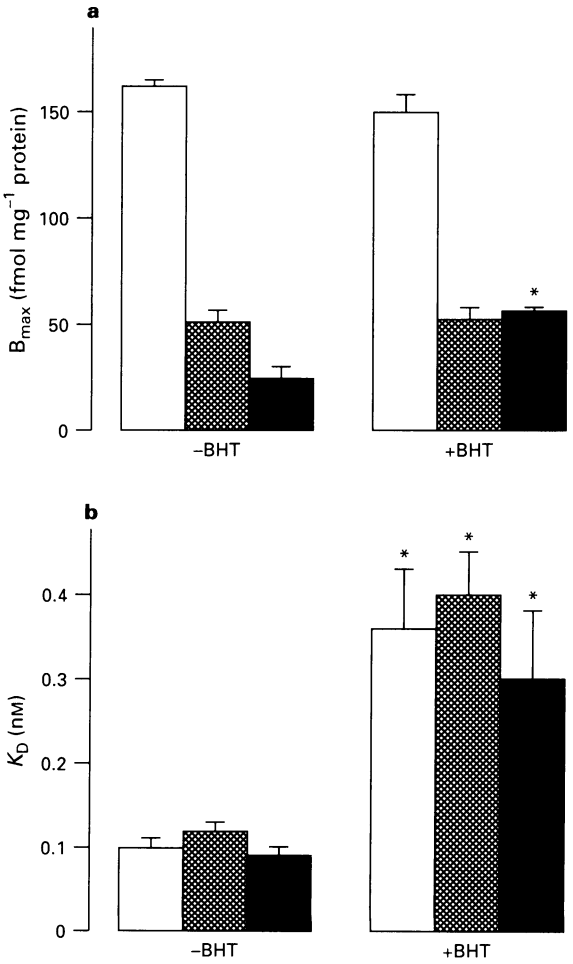

Figure 5 Number (a) and affinity (b) of [^{3}H]-PN200–110 binding sites in control cardiomyocytes (open columns) and in myocytes exposed to the oxygen free radical (OFR)-generating solution for 15 min (cross-hatched columns) or 30 min (solid columns), after preincubation in zero calcium solution (LCS) (−BHT) or in LCS plus the antilipoperoxidant agent butylated hydroxytoluene (50 μM) (+BHT). Data are means s.e.mean of 4–8 experiments. *$P < 0.05$ vs respective values obtained in the absence of BHT.

and 51.0±4.9 fmol mg^{-1} protein in the presence and in the absence of BHT respectively, NS), while it was able to prevent completely the further decrease of [^{3}H]-PN200–110 binding sites occurring during the last 15 min of incubation with OFRs (Figure 5a).

Discussion

In this study we demonstrate that when cardiac ventricular myocytes are exposed to OFRs, the number of DHP binding sites decreases over time, with a rapid decrease during the first 15 min followed by a slower one during the next 15 min. The initial phase is paralleled by a decrease in calcium current, suggesting that the two phenomena are associated. SOD and CAT are able to prevent the effects of OFRs on DHP binding sites and we have previously demonstrated that they also prevent the electrophysiological alterations caused by the same source of free radicals (Cerbai *et al.*, 1991). The late phase, which could not be studied with electrophysiological techniques, is characterized by a slower decrease in DHP binding sites and is prevented by BHT, an antilipoperoxidant agent. The present work consequently extends our knowledge of the interaction of OFR with calcium channels: the reduction in $I_{Ca,L}$ amplitude caused by OFR is due to a direct interaction with the calcium channels and not to indirect mechanisms. We found that [^{3}H]-PN200–110 reveals in ventricular myocytes one population of high affinity binding sites with a B_{max} of 139.1±6.9 fmol mg^{-1} protein. This finding is in agreement with Ptasienski *et al.* (1985) who showed that the presence of either Mg^{2+} or Ca^{2+} in the binding reaction solution results in the disappearance of the lower affinity state and in the conversion to a single high affinity state. Furthermore, the B_{max} value is superimposable on that found in homogenates of rabbit ventricular myocytes (Lew *et al.*, 1991) using the same ligand, which, in isolated myocytes obtained by collagenase treatment, detects only DHP binding sites localised on the sarcolemma (Feron *et al.*, 1992). The number of DHP binding sites is markedly reduced by oxygen free radicals in a time-dependent manner. The generating system is based on dihydroxyfumaric acid (DHF), which is known to generate superoxide, hydrogen peroxide, and the hydroxyl radical (Mak *et al.*, 1983; Kramer *et al.*, 1984), i.e. the relevant reactive oxygen metabolites formed during the reperfusion of the ischaemic myocardium (Bolli *et al.*, 1988; Zweier *et al.*, 1989). DHF has been shown to cause specific electrophysiological alterations in isolated ventricular myocytes when applied intra- or extracellularly (Cerbai *et al.*, 1991; Jabr & Cole, 1993; 1995; Mugelli *et al.*, 1995). The electrophysiological alterations caused by DHF are prevented by scavengers such as SOD, CAT (Cerbai *et al.*, 1991), or mercaptopropionylglycine (Jabr & Cole, 1993). We have previously documented that DHF causes a reduction in $I_{Ca,L}$ amplitude; a similar effect has been shown for other OFR-generating systems (Shattock *et al.*, 1990; Tarr & Valenzeno, 1991; Coetzee & Opie, 1992; Nakaya *et al.*, 1992; Gill *et al.*, 1995). Here we demonstrate that the effect of DHF on $I_{Ca,L}$ amplitude is associated with an almost parallel decrease in DHP binding sites. This phenomenon is clearly observed during the first 10–15 min of exposure to DHF, suggesting a relationship between the number of DHP binding sites and the amplitude of the current. Obviously we are not implying that all the binding sites are functionally active calcium channels. First of all, $I_{Ca,L}$ amplitude is measured in resting conditions, where only a fraction of the calcium channels are activated (i.e. phosphorylated). Second we do not have the proper conditions for measuring the density of calcium channels, which requires the determination not only of the whole-cell current, but also of the single-channel current and the opening probability. However, the similar decreases over time of [^{3}H]-PN200–110 binding sites and of $I_{Ca,L}$ amplitude strongly suggest such a relationship. On the other hand, it has been previously demonstrated by Lew *et al.* (1991) that the density of functional L-type calcium channels (determined from measurement of whole-cell current, single-channel current and opening probability) is comparable to the number of specific DHP receptors (measured using nifedipine-sensitive [^{3}H]-PN200–110 binding). Thus it is not completely surprising that a decrease in $I_{Ca,L}$ amplitude is associated with a decrease in DHP binding sites. What, instead, is important is that an OFR-induced decrease in $I_{Ca,L}$ appears to be fully attributable to a direct effect on calcium channels. This is also confirmed by the measurements of $I_{Ca,L}$ with patch pipettes containing or not containing EGTA: the decrease in current amplitude is similar in all cases, suggesting that $[Ca^{2+}]_i$-dependent inactivation due to OFR-induced calcium overload does not play a major role in the phenomenon. That an increase in $[Ca^{2+}]_i$ occurs, is, however, demonstrated by the appearance of the transient inward current (*TI*) when intracellular calcium is not clamped by EGTA (see Figure 3a). The appearance of *TI* has been previously shown in myocytes exposed to OFRs (Matsuura & Shattock, 1991; Mugelli *et al.*, 1995) and it is well known that *TI* is caused by all the procedures which increase intracellular calcium independently of the mechanism (Vassalle & Mugelli, 1981). It is consequently clear that the increase in cellular calcium loading caused by OFRs is not due to an increase in calcium flux through calcium channels, but involves other pathways. Inactivation by OFRs of the sarcolemmal Ca^{2+} pump, Na^+, K^+-ATPase, and sarcoplasmic reticulum Ca^{2+}-ATPase have been demonstrated (Kim & Akera, 1987; Kaneko *et al.*, 1989a; 1990; Yanagishita *et al.*, 1989; Matsouka *et al.*, 1990; Eley *et al.*, 1991) and all these mechanisms are involved in intracellular calcium homeostasis. Furthermore, here we confirm that OFR cause a decrease in the outward potassium current I_K, which is the main determinant of the prolongation of the action potential duration caused by OFRs (Cerbai *et al.*, 1991). A prolonged plateau can influence intracellular calcium.

The effect of DHF on DHP binding sites is clearly due to the generation of OFRs, since it can be almost completely prevented by the superoxide radical scavenger SOD and the hydrogen peroxide scavenger catalase. These results are in agreement with previous findings showing that different sources of OFRs, such as xanthine-xanthine oxidase (Kaneko *et al.*, 1989b; 1991; Matucci *et al.*, 1992), decrease DHP binding sites in rat membranes without any change in affinity. Since the two scavenging enzymes were used together, we do not have direct evidence of the oxygen radical species involved in the effect. However, previous results indicate that the hydroxyl radical is the main one responsible for the electrophysiological damage caused by DHF (Jabr & Cole, 1993). It has been suggested that oxidative modification of sulphydryl groups of channels or ion transporters (Yanagishita *et al.*, 1989; Matsouka *et al.*, 1990; Eley *et al.*, 1991; Jabr & Cole, 1995) may be involved in the alterations caused by OFRs. It is possible that the decrease in DHP binding sites is due to a similar modification in sulphhydril groups somehow associated with the channel. In this respect, Glossmann *et al.* (1984) have demonstrated that oxidising agents which are able to modify the sulphydryl groups of L-type Ca^{2+} channels decrease the number of DHP binding sites in guinea-pig heart membranes. Furthermore, our data suggest that lipid peroxidation could be involved at least in the late decrease in DHP binding sites induced by OFRs. The antilipoperoxidant agent BHT is able in fact to prevent the decrease in DHP binding sites occurring during the last 15 min of the myocyte exposure to DHF. We do not have direct evidence of the electrophysiological alterations due to lipoperoxidation, such as a further reduction of $I_{Ca,L}$ amplitude, since we were unable to record membrane currents from a single myocyte for more than 10 (in the absence of EGTA into the patch pipette) or 15 min (in the presence of EGTA into the patch pipette) due to cell deterioration as consequence of the exposure to OFRs (namely cell unexcitability, see Cerbai *et al.*, 1991). However it must be recalled that in intact preparations lipid peroxidation is shown to occur with demonstrable changes in electrical activity (Nakaya *et al.*, 1987). Furthermore, it has been shown that *t*-butyl hydroperoxide, a lipoperoxidating agent, reduces $I_{Ca,L}$ in rabbit sinoatrial node (Sato

et al., 1989) and nodal isolated cells (Sato *et al.*, 1995). Interestingly, from a quantitative point of view, the main effect of OFRs on DHP binding sites occurs rapidly, as does the development of electrophysiological effects (Cerbai *et al.*, 1991). These rapid changes are possibly relevant for those alterations, i.e. arrhythmias, associated with the reperfusion of the ischaemic myocardium. However, it is not possible to predict clearly the functional consequences of the decrease in DHP binding sites and $I_{Ca,L}$ amplitude caused by OFRs possibly at the moment of reperfusion. While it is difficult to speculate about development of arrhythmias, which are believed to be the consequence of alterations due to calcium overload (Manning & Hearse, 1984), it is possible that this phenomenon might play a relevant role in the contractile dysfunction associated with the reflow of the ischaemic myocardium. This hypothesis is supported by studies showing that the mechanical dysfunction induced by OFRs is reversed or prevented by sulphydryl group reducing agents (Eley *et al.*, 1989; Ferrari *et al.*, 1991).

In summary, these results clearly demonstrate that the OFR-induced decrease in calcium current is associated with a reduction in DHP binding sites. The latter phenomenon seems to involve both a direct effect on the channel (oxidation of sulphydryl groups?) and lipoperoxidation. The decrease in calcium current and in calcium channels may be implicated in the mechanical dysfunction associated with oxidative stress.

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References

AMBROSIO, G., FLAHERTY, J.T., DUILIO, C., TRITTO, I., SANTORO, G., ELIA, P.P., CONDORELLI, M. & CHIARIELLO, M. (1991). Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. *J. Clin. Invest.*, **87**, 2056–2066.

BARRINGTON, P.L., MEYER, C.F. & WEGLICKI, W.B. (1988). Abnormal electrical activity induced by free radical generating systems in isolated cardiomyocytes. *J. Mol. Cell. Cardiol.*, **20**, 1163–1178.

BERESEWICZ, A. & HORACKOVA, M. (1991). Alterations in electrical and contractile behaviour of isolated cardiomyocytes by hydrogen peroxide: possible ionic mechanisms. *J. Mol. Cell. Cardiol.*, **23**, 899–918.

BOLLI, R. (1988). Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). *J. Am. Coll. Cardiol.*, **12**, 239–249.

BOLLI, R., PATEL, B.S., JEROUDI, M.O., LAI, E.K. & McKAY, P.B. (1988). Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl-N-tert-butyl nitrone. *J. Clin. Invest.*, **82**, 476–485.

BORASO, A. & WILLIAMS, A.J. (1994). Modification of the gating of the cardiac sarcoplasmic reticulum Ca^{2+}-release channel by H_2O_2 and dithiothreitol. *Am. J. Physiol.*, **267**, H1010–H1016.

BURTON, K.P., McCORD, J.M. & GHAI, G. (1984). Myocardial alterations due to free-radical generation. *Am. J. Physiol.*, **246**, H776–H783.

CERBAI, E., AMBROSIO, G., PORCIATTI, F., CHIARIELLO, M., GIOTTI, A. & MUGELLI, A. (1991). Cellular electrophysiological basis for oxygen radical-induced arrhythmias: a patch-clamp study in guinea-pig ventricular myocytes. *Circulation*, **84**, 1773–1782.

CERBAI, E., GUERRA, L., VARANI, K., BARBIERI, M., BOREA P.A. & MUGELLI, A. (1995). β-Adrenoceptor subtypes in young and old rat ventricular myocytes: a combined patch-clamp and binding study. *Br. J. Pharmacol.*, **116**, 1835–1842.

COETZEE, W.A. & OPIE, L.H. (1992). Effects of oxygen free radicals on isolated cardiac myocytes from guinea-pig ventricle: electrophysiological studies. *J. Mol. Cell. Cardiol.*, **24**, 651–663.

ELEY, D.W., ELEY, J.M., KORECKY, B. & FLISS, H. (1991). Impairment of cardiac contractility and sarcoplasmic reticulum Ca^{2+} ATPase activity by hypochlorous acid: reversal by dithiothreitol. *Can. J. Physiol. Pharmacol.*, **69**, 1677–1683.

ELEY, D.W., KORECKY, B. & FLISS, H. (1989). Dithiothreitol restores contractile function to oxidant-injured cardiac muscle. *Am. J. Physiol.*, **257**, H1321–H1325.

FERON, O., WIBO, M., CHRISTEN, M.O. & GODFRAIND, T. (1992). Interaction of pinaverium (a quaternary ammonium compound) with 1,4-dihydropyridine binding sites in rat ileum smooth muscle. *Br. J. Pharmacol.*, **105**, 480–484.

FERRARI, R., CECONI, C., CURELLO, S., CARGNONI, A., ALFIERI, O., PARDINI, A., MARZOLLO, P. & VISIOLI, O. (1991). Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. *Am. J. Med.*, **91**, 95S–105S.

GILL, J.S., MCKENNA, W.J. & CAMM, A.J. (1995). Free radicals irreversibly decrease Ca^{2+} currents in isolated guinea-pig ventricular myocytes. *Eur. J. Pharmacol., (Env. Toxicol. Pharmacol. Section)*, **292**, 337–340.

GLOSSMANN, H., FERRY, D.R., GOLL, A. & RONBUSCH, M. (1984). Molecular pharmacology of the calcium channel: evidence for subtypes, multiple drug-receptor sites, channel subunits and development of a radio-iodinated 1,4-dihydropyridine calcium channel label, [^{125}I]-iodipine. *J. Cardiovasc. Pharmacol.*, **6**, S608–S621.

HEARSE, D.J. (1990). Free radicals, membrane injury, and electrophysiological disorders. In *Cardiac Electrophysiology: From Cell to Bedside*. ed. Zipes, D.P. & Jalife, J. pp. 442–447. Philadelphia, PA: WB Saunders.

HOLMBERG, S.R.M., CUMMING, D.V.E., KUSAMA, Y., HEARSE, D.J., POOLE-WILSON, P.A., SHATTOCK, M.J. & WILLIAMS, A.J. (1991). Reactive oxygen species modify the structure and function of the cardiac sarcoplasmic reticulum calcium-release channel. *Cardioscience*, **2**, 19–25.

JABR, R.I. & COLE, W.C. (1993). Alterations in electrical activity and membrane currents induced by intracellular oxygen-derived free radicals stress in guinea-pig ventricular myocytes. *Circ. Res.*, **72**, 1229–1244.

JABR, R.I. & COLE, W.C. (1995). Oxygen-derived free radicals stress activates nonselective cation current in guinea pig ventricular myocytes. *Circ. Res.*, **76**, 812–824.

KANEKO, M., BEAMISH, R.E. & DHALLA, N.S. (1989a). Depression of the heart sarcolemmal Ca^{2+}-pump activity by oxygen free radicals. *Am. J. Physiol.*, **256**, H368–H374.

KANEKO, M., HAYASHI, H., KOBAYASHI, A., YAMAZAKI, N. & DHALLA, N.S. (1991). Stunned myocardium and oxygen free radicals; Sarcolemmal membrane damage due to oxygen free radicals. *Jap. Circ. J.*, **55**, 885–892.

KANEKO, M., LEE, S.L., WOLF, C.M. & DHALLA, N.S. (1989b). Reduction of calcium channel antagonist binding sites by oxygen free radicals in rat heart. *J. Mol. Cell. Card.*, **21**, 935–943.

KANEKO, M., SINGAL, P.K. & DHALLA, N.S. (1990). Alterations in heart sarcolemmal Ca^{2+}-binding activities due to oxygen free radicals. *Bas. Res. Cardiol.*, **85**, 45–54.

KIM, M-S. AND AKERA, T. (1987). O_2 free radicals: cause of ischemia reperfusion injury to cardiac Na^+-K^+-ATPase. *Am. J. Physiol.*, **252**, H252–H257.

KRAMER, J.H., MAK, T.I. AND WEGLICKI, W.B. (1984). Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical induced lipid peroxidation. *Circ. Res.*, **55**, 120–124.

LEW, W.Y.W., HRYSHKO, L.V. & BERS, D.M. (1991). Dihydropyridine receptors are primarily functional L-type calcium channels in rabbit ventricular myocytes. *Circ. Res.*, **69**, 1139–1145.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MAK, T.I., MISRA, H.P. & WEGLICKI, W.P. (1983). Temporal relationship of free radical induced lipid peroxidation and loss of latent enzyme activity in highly enriched hepatic lysosomes. *J. Biol. Chem.*, **258,** 13733–13737.

MANNING, A.S. & HEARSE, D.J. (1984). Reperfusion-induced arrhythmias: mechanism and prevention. *J. Mol. Cell. Cardiol.*, **16,** 497–518.

MATSOUKA, T., KATO, M. & KAKO, K.J. (1990). Effect of oxidants on Na, K, ATPase and its reversal. *Basic Res. Cardiol.*, **85,** 330–341.

MATSUURA, H. & SHATTOCK, M.J. (1991). Membrane potential fluctuations and transient inward currents induced by reactive oxygen intermediates in isolated rabbit ventricular cells. *Circ. Res.*, **68,** 319–329.

MATUCCI, R., OTTAVIANI, M.F., CAMPANA, S., BIANCHI, B., BENNARDINI, F., MONETI, G., GIOTTI, A. & FRANCONI, F. (1992). Do ethoxy radicals reduce ^{3}H-nitrendipine binding in rat cardiac membranes? *Pharmacol. Res.*, **26,** 151–159.

MCCORD, J.M. (1988). Free radicals and myocardial ischemia: overview and outlook. *Free Rad. Biol. Med.*, **4,** 9–14.

MIKUNI, T., TATSUDA, M. & KAMACHI, M. (1987). Scavenging effect of butylated hydroxytoluene on the production of free radicals by the reaction of hydrogen peroxide with N-methyl-N′-nitro-N-nitrosoguanidine. *J. Natl. Cancer Inst.*, **79,** 281–283.

MUGELLI, A., CERBAI, E., BARBIERI, M. & LI, Q. (1995). Cellular electrophysiological aspects of myocardial protection. *Pharmacol. Res.*, **31,** 243–249.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerised approach for the characterization of ligand binding systems. *Anal. Biochem.*, **107,** 220–239.

NAKAYA, H., TAKEDA, Y., TOHSE, N. & KANNO, M. (1992). Mechanism of the membrane depolarization induced by oxidative stress in guinea-pig ventricular cells. *J. Mol. Cell. Cardiol.*, **24,** 523–534.

NAKAYA, H., TOHSE, N. & KANNO, M. (1987). Electrophysiological derangements induced by lipid peroxidation in cardiac tissue. *Am. J. Physiol.*, **253,** H1089–H1097.

PTASIENSKI, J., MCMAHON, K.K. & HOSEY, M.M. (1985). High and low affinity states of the dihydropyridine and phenylalkylamine receptors on the cardiac calcium channel and their interconversion by divalent cations. *Biochem. Biophys. Res. Commun.*, **129,** 910–917.

SAKAMOTO, A., OHNISHI, S.T., OHNISHI, T. & OGAWA, R. (1991). Relationship between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain. *Brain Res.*, **554,** 186–192.

SATO, N., NISHIMURA, M., TANAKA, H., HOMMA, N. & WATANABE, Y. (1989). Augmentation and subsequent attenuation of Ca^{2+} current due to lipid peroxidation of the membrane caused by t-butyl hydroperoxide in the rabbit sinoatrial node. *Br. J. Pharmacol.*, **98,** 721–723.

SATO, N., NISHIMURA, M. & WATANABE, Y. (1995). Electrophysiologic alterations in the rabbit nodal cells induced by membrane lipid peroxidation. *Eur. J. Pharmacol., (Env. Toxicol. Pharmacol. Section)*, **292,** 233–240.

SHATTOCK, M.J., HEARSE, D.J. & MATSUURA, H. (1990). Ionic currents underlying oxidant stress-induced arrhythmias. In *Ionic Currents and Ischemia.* ed. Vereecke, J., van Bogaert, P.P. & Verdonck, F. pp. 288–290. Leuven: University Press.

SHATTOCK, M.J. & MATSUURA, H. (1993). Measurement of Na^+K^+-pump current in isolated rabbit ventricular myocytes using the whole-cell voltage clamp technique. *Circ. Res.*, **72,** 91–101.

STANFIELD, P.R. (1986). Voltage-dependent calcium channels of excitable membranes. *Br. Med. Bull.*, **42,** 359–367.

TARR, M. & VALENZENO, D.P. (1991). Modification of cardiac ionic currents by photosensitizer-generated reactive oxygen. *J. Mol. Cell. Cardiol.*, **23,** 639–649.

VASSALLE, M. & MUGELLI, A. (1981). An oscillatory current in sheep cardiac Purkinje fibers. *Circ. Res.*, **48,** 618–631.

YANAGISHITA, T., MATSOUKA, T. & KAKO, K.J. (1989). Pharmacological intervention in oxidant-induced calcium pump dysfunction of dog heart. *Biochem. Int.*, **18,** 1111–1119.

ZWEIER, J.L., KUPPUSAMY, P., WILLIAMS, R., RAYBURN, B.K., SMITH, D., WEISFELDT, M.L. & FLAHERTY, J.T. (1989). Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *J. Biol. Chem.*, **264,** 18890–18895.