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ORIGINAL ARTICLE

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The influence of oxidative stress on various inotropic responses in isolated rat left atria

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Abstract The effects of free radicals, generated by electrolysis of a physiological salt solution, on various inotropic responses to drugs in isolated rat left atria were studied. Evidence for the generation of hydroxyl radicals was obtained from an appropriate fluorimetric assay. The amount of free radicals produced by electrolysis of the medium proved current-dependent. Exposure of isolated rat left atria to the medium which had been subjected to electrolysis caused a current-dependent decrease in contractile force. Oxidative stress, as a result of the electrolysis of the medium, caused altered inotropic responses to extra cellular Ca^{2+} (pD_2 control group: 2.62 ± 0.06 vs. 2.44 ± 0.07 electrolysis group), sodium withdrawal (rise in contractile force control group: 1.73 ± 0.19 mN vs. 0.48 ± 0.21 mN electrolysis group) and lowering of stimulation frequency. The response to isoprenaline was diminished in atria subjected to oxidative stress and led to a rightward shift of the concentration response curves (pD_2 control group: 7.56 ± 0.10 vs. 6.77 ± 0.11 electrolysis group). In addition, the inotropic responses to forskolin (pD_2 control group: 6.17 ± 0.12 vs. < 4.5 electrolysis group) and dibutyryl cAMP (rise in contractile force caused by 1×10^{-5} M db-cAMP in control group: 2.15 ± 0.01 mN vs. 1.21 ± 0.10 mN electrolysis group) proved blunted as well. Measurement of the adenylyl cyclase activity revealed that free radicals attenuated the basal (by 11.1%) and forskolin stimulated (155.0 ± 5.1 vs. 48.0 ± 1.8 pmol cAMP/mg prot./min for control and electrolysis group respectively) activity of the adenylyl cyclase. DMSO, a well known hydroxyl radical scavenger, was able to abolish the free radical-induced decrease in the response to isoprenaline.

Surprisingly, addition of α-adrenoceptor agonists to atria subjected to electrolysis-generated free radicals led to a rapid decrease in contractile force. DMSO was unable to counteract the negative intropic effect of methoxamine in atria subjected to oxidative stress. This negative inotropic response to α-adrenoceptor agonists in atria subjected to electrolysed medium is unlikely to be the direct result of phospholipase C or protein kinase C activation. Angiotensin II (which stimulates PLC as well) did not reduce contractile force and chelerythrine (a PKC inhibitor) was unable to counteract the negative inotropic effect of the adrenoceptor agonists. In addition, the negative inotropic effect of methoxamine proved insensitive to 10^{-6} M phentolamine and 10^{-5} M doxazosin, which indicates an α-adrenoceptor independent mechanism.

From this study we conclude that free radicals alter responses to various inotropic stimuli. These alterations may be the result of injured contractile elements, transporter molecules and molecules involved in signal transduction. Addition of α-adrenoceptor agonists after oxidative stress leads to a α-adrenoceptor, PLC and PKC independent decrease in contractile force.

Key words Free radicals · Electrolysis · Adrenoceptors · Heart · Inotropic responses

Introduction

Oxygen derived free radicals (OFR) play a major role in the pathology of ischaemia/reperfusion injury (Zweier et al. 1987; Baker et al. 1988) and in many other disease states (Nakazono et al. 1991; Tesfamariam and Cohen 1992; Witztum 1994). OFR are produced continuously in vivo and they are normally inactivated by enzymes like superoxide dismutase, catalase and glutathion peroxidase/reductase and by well known antioxidants like vitamine E and C, β-carotene and uric acid (Fridovich 1974; McCay 1985; Halliwell et al. 1992). In certain pathological situations, the antioxidant capacity of cells may be too small to scavenge the excess of free radicals produced, which subsequently leads to cellular injury (Ferrari et al. 1985). Be-

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cause of their high reactivity free radicals can rapidly oxidize different biological molecules such as lipids, nucleic acids and proteins. During the reperfusion phase after ischaemia, an excess of OFR in the myocardium can lead to lipid peroxidation and alterations in important cardiac structures such as adrenoceptors, ion-channels, enzymes and contractile proteins (Freeman and Crapo 1982; Meerson et al. 1982; Kaneko et al. 1989 and 1991; Prasad et al. 1989; Semb et al. 1989), thus inducing a decrease in contractile force. Extensive research has been performed on adrenoceptor stimulation after ischaemia/reperfusion, providing many indirect data concerning the effects of free radicals on adrenoceptor function. These studies indicate that ischaemia and reperfusion lead to an impaired inotropic responsiveness of the heart to adrenoceptor agonists (Meggs et al. 1992; van den Ende et al. 1994). So far the influence of free radicals on the inotropic effects of α- and β-adrenoceptor agonists and other agents that enhance cardiac contractile force has been studied but superficially. In these few studies free radicals clearly changed the molecular characteristics of the adrenoceptors, as reflected by altered binding profiles and upregulation of α- and β-adrenoceptors. Similarly, impaired responses to adrenergic stimulation were established (Haenen et al. 1988, 1990; Kaneko et al. 1991). It was the aim of the present study to investigate the functional, pharmacological and biochemical changes in myocardial tissue as a result of oxidative stress in more detail. For this purpose we developed a suitable method to induce oxidative stress, which may also be used for the investigation of anti-oxidant agents.

Materials and methods

Isolated left atria. Male Wistar rats (Iffa Credo, Les Oncins, France), weighing 260–300 g, were sacrificed by stunning and decapitation. The hearts were removed quickly and placed in a physiologic salt solution (PSS) of the following composition (in mM): NaCl 119; KCl 4.5; $MgCl_2$ 0.5; $CaCl_2$ 2.5; glucose 11; Tris 30 (pH 7.5, room temperature), and bubbled with 100% oxygen. The isolated left atria were suspended in water jacketed organ baths (thermostated at 37°C, and gassed with 100% O_2) filled with 5 ml PSS and connected with a silk thread to a Kyowa force transducer. The atria were paced with a field stimulator (Hugo Sachs Electronic, Germany) at a frequency of 3 Hz. The isometric force was recorded on a WKK device. The resting tension was adjusted to 5 mN and the atria were allowed to equilibrate for at least 45 min. At 30 min intervals the medium was exchanged against fresh buffer solution.

Electrolysis of the medium. After the equilibration period free radicals were generated by electrolysis of the medium by means of two additional platinum wire electrodes (each 0.75 cm in length) circular at the bottom of the organ baths (∅ 1.4 cm). Free radical generation by electrolysis of a physiological salt solution was first described by Jackson et al. (1986) and the procedure was modified for the exposure of isolated atria to electrolysed medium in the present study. For this purpose a constant current of 30 mA, generated by a 6 channel constant current device (dept. of electronics, Academic Medical Centre Amsterdam), was applied for 75 s.

Direct and indirect free radical assays. In order to investigate whether the electrolysis indeed leads to the generation of free radicals, control experiments with fluorescense probes and free radical scavengers were performed.

a) In control experiments free radical scavengers were added 15 min before electrolysis to the medium and they were present during the rest of the experiment. Time/force relations of the atria were followed for 60 min for both electrolysis and electrolysis/scavenger groups. The following scavengers were used: Dimethylsulfoxide (DMSO) 10 mM (a well known hydroxyl radical scavenger), superoxide dismutase (SOD) 100 $U \cdot ml^{-1}$ (converts superoxide anions to H_2O_2) catalase 150 $U \cdot ml^{-1}$ (converts H_2O_2 to $H_2O + O_2$), a combination of SOD (100 $U \cdot ml^{-1}$) catalase (150 $U \cdot ml^{-1}$) and ascorbic acid 50 mM (a non-selective reductant and lipid peroxidation inhibitor).
b) Terephtalic acid (TPA) (para-carboxy benzoate) was used as a hydroxyl radical specific fluorescent probe in control experiments without atria in the organ bath. Terephthalic acid (non-fluorescent) can be hydroxylated by hydroxyl radicals to yield a fluorescent product (monohydroxyterephthalate) (Barreto et al. 1995), which can be determined quantitatively. TPA (10 mM) was dissolved in the normal buffer solution (pH 7.5) and constant currents of 5, 15 and 30 mA, respectively, were applied for periods of 75 s. The fluorescense was measured with a Shimadzu RF-5001 PC spectrofluorometer (excitation 312 nm, emission 426 nm). The effects of the hydroxyl radical scavengers DMSO (100 mM) and mannitol (100 mM) were measured as well.

Inotropic drugs. Cumulative concentration response curves for isoprenaline ($1 \cdot 10^{-9}$–$3 \cdot 10^{-5}$ M), forskolin ($3 \cdot 10^{-7}$–$3 \cdot 10^{-4}$ M), methoxamine ($3 \cdot 10^{-5}$–$3 \cdot 10^{-2}$ M), cirazoline ($1 \cdot 10^{-7}$–$3 \cdot 10^{-3}$ M), ST 587 ($1 \cdot 10^{-7}$–$3 \cdot 10^{-3}$ M) and calcium ions ($1 \cdot 10^{-3}$–$1 \cdot 10^{-2}$ M) were constructed 30 min subsequent to the period of electrolysis, without changing the medium. Because of the slow onset of the effect, the positive inotropic action of dibutyryl cAMP was measured at a single concentration ($1 \cdot 10^{-5}$ M) only. In addition the inotropic effects of lowering frequency and sodium concentration (119 > 40 mM) were measured. The protective effect of DMSO (10 mM) was investigated in connection with the experiments with isoprenaline and methoxamine.

Adenylyl cyclase assay. The effects of free radicals on adenylyl cyclase activity were determined in vitro by using left rat atrium membrane preparations. Six membrane suspensions were prepared; three of control atria and three of atria subjected to electrolysis. For each group 3 atria (about 70 mg) were pooled. Organs were rapidly frozen in liquid nitrogen and stored at –80°C for not longer than one day before use in the assay. The atria were minced with scissors in 5 ml ice-cold 1 mM $KHCO_3$ solution, and homogenized with an Ultraturrax (full speed 10 s and twice 20 s at 2/3 speed). The homogenate was filtered through two layers of cloth gauze and centrifuged at 40000 g for 20 min. The pellet containing the membrane fraction was resuspended and rehomogenized in 1 ml TEN buffer (30 mM Tris, 1 mM EDTA, 25 mM NaCl, pH 7.4). The protein concentration in the membrane suspension was assessed by the method of Bradford (1976) using bovine serum albumine as a standard and adjusted to 0.5 mg protein$\cdot ml^{-1}$. For the determination of adenylyl cyclase activity, a 20 µl aliquot of the membrane suspension was added to the incubation tubes (6×), containing 10 µl of a cyclase buffer (400 mM HEPES, 50 mM $MgCl_2$, 10 mM EDTA, pH 7.4), 10 µl of a 20 $U \cdot ml^{-1}$ adenosine deaminase suspension, 10 µl of 10^{-5} M forskolin solution or 10 µl distilled water (basal) and (final incubation concentrations) 50 $U \cdot ml^{-1}$ creatine phosphokinase, 0.5 mM ATP, 2 µM GTP and 5 mM creatine phosphate, 1 mM isobutylmethylxanthine. The final volume in each assay tube was 100 µl.

After 5 min of pre-incubation at 30°C, the membrane suspension was added and the incubation was continued for an additional 10 min. The reaction was stopped by placing the tubes in boiling water for 4 min. The tubes were then centrifuged at 2000 g for 15 min. The cAMP content of 20 µl supernatant was quantitated by means of a commercially available protein binding assay (Amersham TRK 432). The basal and forskolin stimulated adenylyl cyclase activities were expressed as pmol cAMP/mg protein/min.

Statistics. The data are expressed as means ± SEM. Student's *t*-test and ANOVA (Dunnet) were used and p values < 0.05 were considered to be statistically significant.

Chemicals. (–) Isoprenaline bitartrate, dibutyryl cAMP sodium salt, methoxamine HCl, guanosine triphosphate, adenosine deaminase, creatine phosphokinase, creatine phosphate, isobutylmethylxanthine, angiotensin II, phentolamine and terephthalic acid were purchased from Sigma Chemical, St Louis, MO, USA. Catalase, superoxide dismutase and adenosine triphosphate were purchased from Boehringer Mannheim, Germany, dimethyl sulfoxide from Janssen Chimica, Beerse, Belgium, L-ascorbic acid from Merck, Darmstadt, Germany and chelerythrine chloride from ICN Biomedicals, Zoetermeer, The Netherlands. Forskolin was kindly donated by Hoechst, Amsterdam, The Netherlands, cirazoline HCl by Synthélabo, Paris, France and ST 587 (2-chloro-5-trifluoro-methylphenylimino-2-imidazolidine) by Boehringer Ingelheim KG, Germany.

Results

Formation of free radicals

After exposition of the medium to electrolysis for 75 s a current-dependent increase in fluorescence was observed in the terephthalate fluorescence assay, indicating the generation of monohydroxy terephthalate as a result of free radical formation (Fig. 1). Because the hydroxylation of terephthalic acid is selective for hydroxyl radicals, this experiment indicates the formation of hydroxyl radicals only. DMSO (100 mM) and mannitol (100 mM) were both able to reduce the formation of monohydroxyterephthalate after electrolysis (30 mA) by 79% ($p<0.05$) and 45%, ($p<0.05$) respectively.

Influence of electrolysis of the medium on contractile force

Exposure of isolated left atria to the medium which had been subjected to electrolysis caused a current-dependent decrease in contractile force (initial force of contraction: 8.2 ± 0.32 mN). The time course of these effects is shown in Fig. 2A, indicating a gradual decrease of contractile force which had not reached equilibrium after 1 h. Electrolysis of the medium did not influence the stimulation-induced frequency of beating.

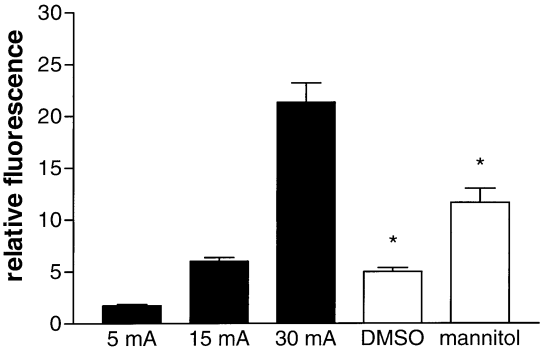

Fig. 1 Electrolysis-induced hydroxylation of terephthalic acid, indicating the current-dependent formation of hydroxyl radicals. Constant currents of 5, 15 and 30 mA were applied for 75 s. In experiments with DMSO (100 mM) and mannitol (100 mM), a constant current of 30 mA was applied for 75 s. Note the suppressant effects of DMSO and mannitol on radical formation. Points represent means ± SEM, (n = 6). * $p<0.05$ compared to 30 mA electrolysis

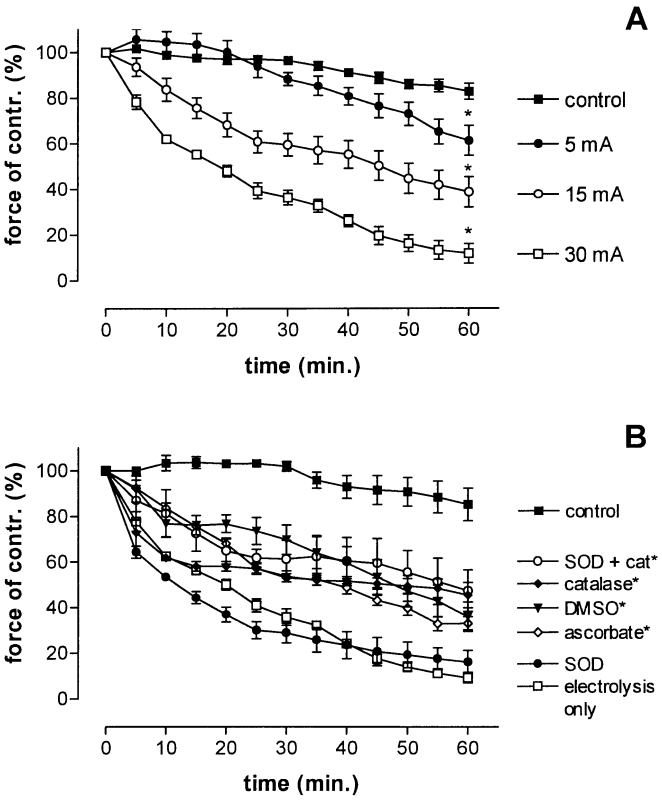

Fig. 2A Influence of preceding electrolysis of the medium on the contractile force of paced (3 Hz) isolated rat left atria. Currents were applied for 75 s. Points represent means ± SEM, (n = 6–8). * $p<0.05$ compared to next curve. **B** Influence of free radical scavengers on free radical induced decrease in contractile force of isolated rat left atria. Scavengers were added to the medium 15 min prior the period of electrolysis (30 mA, 75 s) and they were present during the rest of the experiment. Points represent means ± SEM, (n = 4–6). * $p<0.05$ compared to electrolysis only

DMSO (10 mM), ascorbic acid (50 mM), catalase (150 $U \cdot ml^{-1}$) and a combination of catalase (150 $U \cdot ml^{-1}$) and SOD (100 $U \cdot ml^{-1}$) were able to counteract the reduction in contractile force induced by electrolysis of the bath fluid. However, SOD alone (100 $U \cdot ml^{-1}$) proved ineffective in this respect (Fig. 2B).

Inotropic drugs

Isoprenaline, forskolin and dibutyryl cAMP. Prior electrolysis of the medium induced significant and substantial rightward shifts of the concentration response curves for the inotropic responses to isoprenaline (pD_2: 7.56 ± 0.10 to 6.77 ± 0.11, $p<0.05$) and forskolin, which directly activates adenylyl cyclase (pD_2: 6.17 ± 0.12 to < 4.5) (Fig. 3A,B). The inotropic response to dibutyryl cAMP also proved diminished after electrolysis of the medium (Fig. 3D): the increase in contractile force to $1 \cdot 10^{-5}$ M dibutyryl cAMP amounted to 2.15 ± 0.01 mN under control conditions and to 1.21 ± 0.10 mN after electrolysis of the bath fluid, respectively ($p<0.05$).

Measurements in membrane suspensions indicated that the basal activity of the adenylyl cyclase in atria subjected

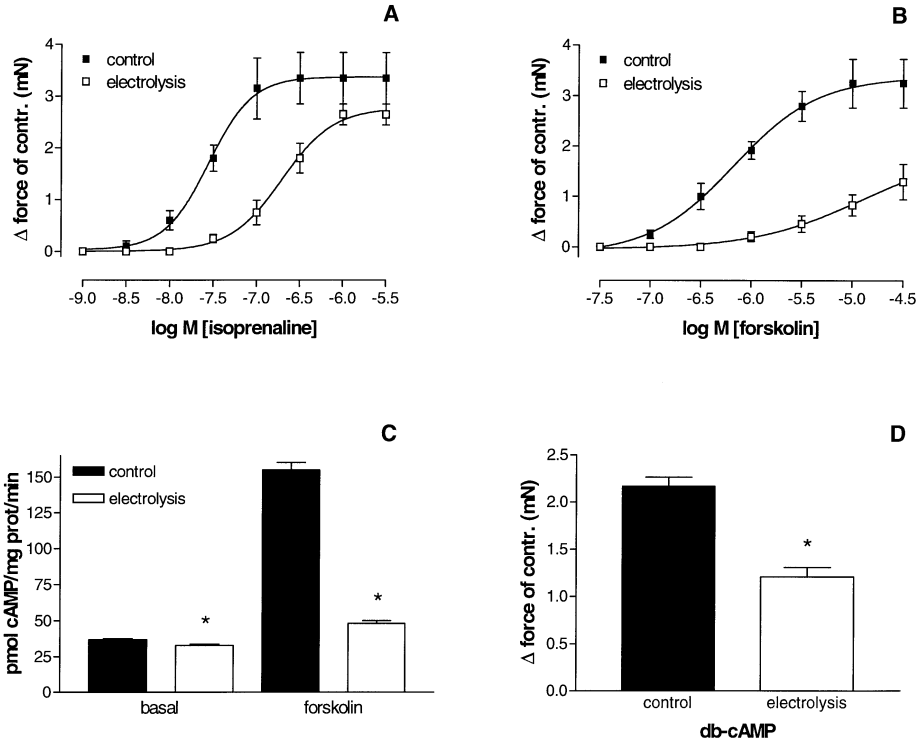

Fig. 3 Concentration response curves for the effects of **A** isoprenaline and **B** forskolin in control atria (*closed squares*) and in atria exposed for 30 min to electrolyzed (30 mA, 75 s) medium (*open squares*). Points represent means ± SEM, (n = 5–8). $p<0.05$ for pD_2 compared to controls. **C** Basal and forskolin stimulated adenylyl cyclase activity in control atria (*closed bars*) and atria subjected to electrolyzed medium (*open bars*). Measurements were performed six times in three homogenates of three pooled atria per group. Values given as means ± SEM. **D** Effect of $1 \cdot 10^{-5}$ M dibutyryl cAMP on contractile force in control atria and in atria subjected to oxidative stress. Dibutyryl cAMP was added to the medium 30 min after electrolysis (n = 4–6). * $p<0.05$ compared to control

to oxidative stress was decreased by 11.1% when compared with control organs ($p<0.05$). In addition, the amount of cAMP formed in response to the direct stimulation of adenylyl cyclase with 10^{-6} M forskolin proved significantly decreased in atria exposed to free radicals: 155.0 ± 5.1 versus 48.0 ± 1.8 pmol cAMP/mg protein/min for control and electrolysis groups, respectively (Fig. 3C).

Because the addition of DMSO to the medium precluded the construction of a cumulative concentration response curve for isoprenaline, the protective effect of 10 mM DMSO was tested for a concentration of $1 \cdot 10^{-5}$ M isoprenaline only. Without DMSO in the medium the rise in contractile force to isoprenaline was 2.7 ± 0.20 mN (control) versus 1.7 ± 0.15 mN after electrolysis of the medium ($p<0.05$). DMSO was able to abolish the free radical-induced decrease in the response to isoprenaline (data not shown).

Methoxamine, cirazoline and ST-587

Figure 4A shows the concentration response curve for the inotropic effect of methoxamine. The pD_2 values for methoxamine in control atria amounted to 4.23 ± 0.09 (pD_2 values for cirazoline and ST-587 amounted to 4.93 ± 0.18 and 4.59 ± 0.17, respectively). In atria incubated in a medium subjected to electrolysis, methoxamine surprisingly induced a rapid decrease in contractile force. Similar effects were observed for the α-adrenoceptor agonists cirazoline and ST-587 (data not shown). To examine whether this decrease in contractile force following α-adrenoceptor agonist addition is more pronounced than the usual decrease observed after electrolysis, we compared the time course of atria subjected to oxidative stress and stimulation with the three α-adrenoceptor agonists to atria exposed to oxidative stress only (Fig. 4B–D). In these experiments agonist concentrations were used that caused a maximal inotropic response in control atria ($3 \cdot 10^{-4}$ M methoxamine, $3 \cdot 10^{-4}$ M cirazoline and $1 \cdot 10^{-4}$ M ST-587). All three α-adrenoceptor agonists induced a negative inotropic response in atria exposed to bath fluid subjected to electrolysis. Most atria in the electrolysis group stopped beating within 15 min after administration of methoxamine to this medium. Addition of DMSO (10 mM) did not prevent the negative inotropic effect of methoxamine in atria subjected to oxidative stress. However, in the presence of DMSO, methoxamine induced a transient (± 2 min) positive inotropic effect. After this short period a rapid decrease in contractile force was seen comparable to the effect without DMSO in the medium (data not shown). The negative inotropic effect of methoxamine proved insensitive to phentolamine (10^{-6} M) and doxazosin (10^{-5} M) which may indicate an α-adrenoceptor independent mechanism. To investigate whether phospholipase C (PLC) and/or proteine kinase C (PKC) may play a role in the negative inotropic effect of α-adrenoceptor agonists after oxidative stress, we also studied the effects of angiotensin II and chelerythrine. Angiotensin II, which causes a chronotropic effect in rat atria predominantly via the stimulation of PLC, did not influence contractile force in the stimulated left rat atria, neither in the control group nor in the electrolysis group. In addition, the protein kinase C inhibitor chelerythrine (2 μM) was unable to counteract the negative inotropic effects of methoxamine (data not shown).

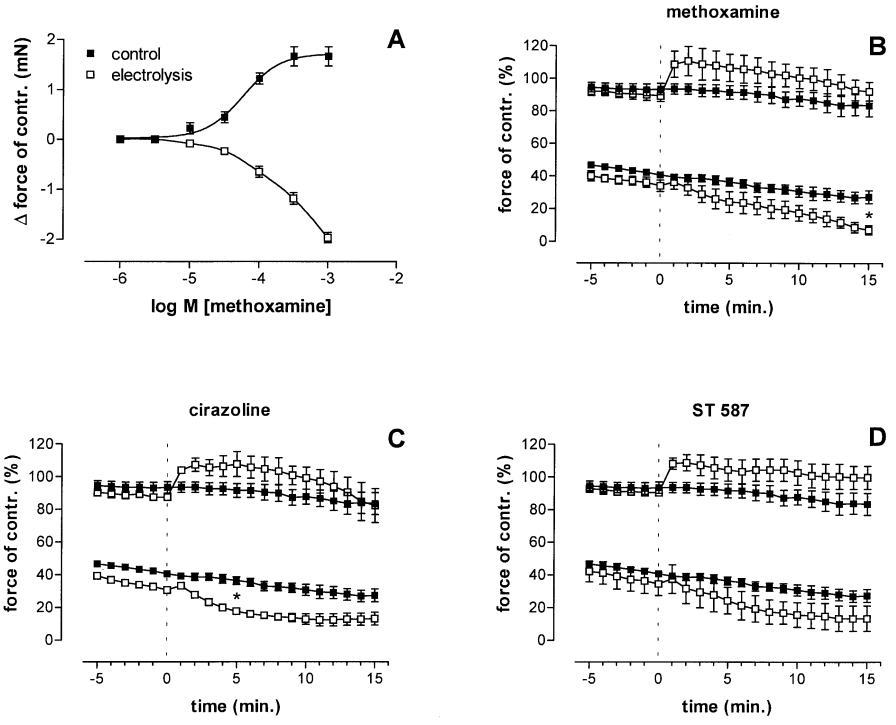

Fig. 4A Concentration response curve for the effects of methoxamine in control atria (*closed squares*) and in atria subjected to oxidative stress (*open squares*) induced by electrolysis (30 mA, 75 s). Points represent means ± SEM, (n = 4–6). **B, C** and **D** Time course relationship for methoxamine ($3 \cdot 10^{-4}$ M), cirazoline ($3 \cdot 10^{-4}$ M) and ST-587 ($1 \cdot 10^{-4}$ M) induced inotropic responses in control atria (*upper curves, open squares*) and atria subjected to oxidative stress (*lower curves, open squares*). For comparison, curves for atria (control; *upper curves* electrolysis; *lower curves*) without pharmacological intervention are given (*closed squares*). Agonists were added to the medium 30 min subsequent to the period of electrolysis (*dotted line*). Points represent means ± SEM, (n = 3). * p<0.05 compared to electrolysis only

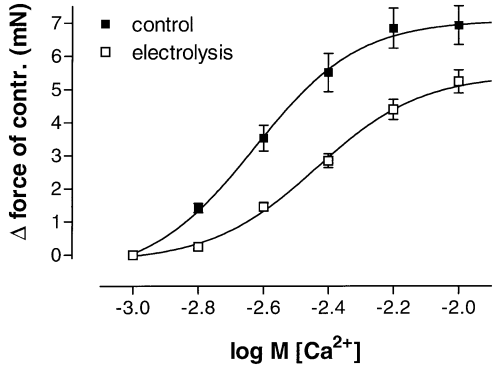

Fig. 5 Concentration response curves for extracellular calcium ions in control atria (*closed squares*) and in atria subjected to oxidative stress (*open squares*) by means of electrolysis of the medium (30 mA, 75 s). Points represent means ± SEM, (n = 6). p<0.05 for pD_2 compared to control

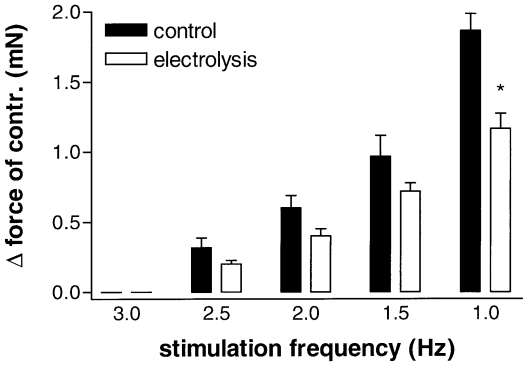

Fig. 6 Positive inotropic effect in control atria and in atria subjected to oxidative stress, as a result of lowering stimulation frequency (*inversed Bowditch staircase*). Values given as means ± SEM, (n = 6). * p<0.05 compared to control

Other inotropic stimuli

The isolated left atria subjected to an electrolysed medium showed a diminished response to extracellular calcium, with pD_2 values of 2.62 ± 0.06 versus 2.44 ± 0.07 for the control and electrolysis group, respectively (p<0.05) (Fig. 5). Lowering the sodium concentration from 119 to 40 mM, which leads to a calcium influx via the sodium/calcium exchanger, resulted in an inotropic response which was more pronounced in control atria (1.73 ± 0.19 mN) than in organs exposed to an electrolysed bath fluid (0.48 ± 0.21 mN, p<0.05) (data not shown). In this experiment normal osmolality was preserved by the addition of 158 mM saccharose. In the rat myocardium the frequency-force relationship is known to be inversed when compared with other species (the Bowditch staircase). Accordingly, the lowering of the frequency of beating induces a positive inotropic response. In atria subjected to oxidative stress this phenomenon persisted, although the relative increase in contractile force proved less pronounced (Fig. 6).

Discussion

It is well established that oxygen derived free radicals play an important role in the etiology of ischaemia/reperfusion injury. Several free radical producing systems have been used to study the effects of free radicals on various tissues. These systems include for instance; xanthine/xanthine oxidase, hydrogen peroxide, hydrogenperoxide/Fe(II), dihydroxy fumarate and several organic radicals like cumene hydroperoxide and 4-hydroxynonenal. Free radical generation by electrolysis of a physiological salt

solution was first described by Jackson et al. (1986). This system has the advantage that a wide range of free radicals are produced without addition of enzymes or chemicals which themselves may influence the experiment. Electrolysis is rather easy to perform and cheap. Different groups have shown in a direct or indirect manner that the electrolysis system used generates superoxide anions, hydroxyl radicals, hydrogen peroxide, singlet oxygen and hypochlorite (Jackson et al. 1986; Chahine et al. 1991; Niu et al. 1995; de Keulenaer et al. 1995). In the present study we were unable to detect superoxide anions by means of a cytochrome C reduction spectrophotometric assay. In addition, superoxide dismutase did not show any protective effect in the functional studies (Fig. 2B). These findings imply that, under the conditions used, superoxide anions are not generated, or that the superoxide anions are rapidly converted into other free radical species. The terephthalate fluorimetric assay clearly demonstrates the formation of hydroxyl radicals since hydroxylation of terephthalic acid is specific for hydroxyl radicals (Barreto et al. 1995). Moreover DMSO and mannitol (both specific hydroxyl radical scavengers) were able to prevent the formation of the fluorescent monohydroxy terephtalate in this assay. The protective effect of 10 mM DMSO in the functional studies is in accordance with this finding. Similarly the protective effects of catalase in the experiments with isolated left atria demonstrate the formation of hydrogen peroxide during the electrolysis period, which may be generated via the conversion of other types of radicals.

Although the addition of glycine (a known scavenger of hypochlorite) had no effect in our functional studies, it may be possible that hypochlorite is also formed by electrolysis of a physiological salt solution (Jackson et al. 1986). De Keulenaer et al. (1995) have shown, by means of a fluorimetric assay, that the concentration of free radicals during electrolysis is constant in time because of the extremely short half life of the hydroxyl radical and superoxide anions (milliseconds to seconds), indicating that there exists an on/off situation (no free radicals were detectable directly after the period of electrolysis). Their and our results show in a direct and indirect way that the concentration of free radicals during electrolysis is current-dependent. Accordingly, the amount of free radicals to which the atria will be exposed can be easily regulated by varying the current and/or time of electrolysis. Electrolysis of the medium led to a gradual decrease in contractile force in the isolated left atria exposed to this medium. This decrease in contractile force is most likely the result of alterations in contractile elements and/or a disturbed calcium handling in the myocyte, which may subsequently lead to a calcium overload. It has been shown that free radicals can depress the activity of the sarcolemmal Ca^{2+}-pump (Kaneko et al. 1989), which is involved in the efflux of calcium from the myocyt, and the sarcoplasmic reticular Ca^{2+}-pump (Rowe et al. 1983), which plays a role in the sequestration of calcium ions into the sarcoplasmic reticulum. In this connection it also has been shown that free radicals may modulate Ca^{2+}-(leak) channels (Kaneko et al. 1989), as well as the Na^{+}/Ca^{2+} exchanger (Tani 1990; Clague et al. 1993; Wang et al. 1995). The altered response to sodium withdrawal in atria exposed to free radicals in the present study may reflect alterations in the Na^{+}/Ca^{2+} exchanger. These changes in important ion transport molecules will decrease the efflux of calcium from the myocyte, thus leading to a condition of calcium overloading. In the present study the response to extracellular calcium proved diminished after free radical exposure. The rightward shift of the concentration response curve and lower E_{max}, may be the result of injury to calcium transport molecules and/or to injured contractile elements.

In the rat heart the frequency/force relationship is inversed (the negative Bowditch staircase) when compared to hearts from other species, including humans (Hoffman and Kelly 1959). Accordingly, lowering of frequency of beating in isolated left atria of the rat will result in a positive inotropic response. Several hypotheses regarding the mechanism of this effect in the rat myocardium have been proposed. Recent research by Field et al. (1996) revealed that increasing of frequency leads to an elevation of end-diastolic intra-cellular Ca^{2+} and a decrease in ventricular pressure. These authors proposed that the inability of the sarcoplasmic reticulum to sequester sufficient cytosolic calcium at higher stimulation frequencies leads to an elevation in end-diastolic intracellular Ca^{2+}, a decreased net calcium flux per cycle, thus resulting in a negative intracellular Ca^{2+} staircase and concomitantly a negative inotropic response. In our experiments the positive inotropic response after lowering the stimulation frequency proved to persist after exposure to free radicals, although the rise in contractile force was smaller when compared to control atria. This finding indicates that the sequestration capability of the myocytes after free radical exposure is largely intact.

Oxidative stress has been reported to reduce the inotropic response to β-adrenoceptor stimulation (Haenen et al. 1988, 1990). This reduction in inotropic response may be the result of a modification of the receptor itself, of the G-protein, or of the effector adenylyl cyclase. In addition, oxidative injury to other subcellular structures such as contractile proteins, enzymes and membrane components may also (partially) account for the altered response to adrenoceptor stimulation. A few studies have shown that oxygen derived free radicals can modify adrenoceptors in cardiac tissue, thus resulting in altered binding characteristics and upregulation of the receptors (Haenen et al. 1990; Kaneko et al. 1991). The reports on changes in signal transduction, induced by ischaemia and reperfusion are contradictionary and largely depend on the species used, the duration of ischaemia and/or reperfusion and the method used to provoke ischaemia. Anti-oxidants in these studies have shown beneficial effects (for review see Opie 1989). Conflicting results have been obtained regarding changes in receptors, G-proteins and adenylyl cyclase activity. In the present study we found that free radicals induce a diminished response to isoprenaline and forskolin, and reduce adenylyl cyclase activity (Fig. 4). However, the contribution of these alterations in signal transduction to the reduced response to β-adrenoceptor stimulation is questionable, be-

cause dibutyryl cAMP itself also proved less effective. In addition, atria subjected to oxidative stress also show an impaired response to sodium withdrawal and lowering of frequency. Accordingly, it seems likely that the injury of other subcellular components may play an important role in the blunted responses to β-adrenoceptor stimulation.

Stimulating atria subjected to oxidative stress with α-adrenoceptor agonists surprisingly led to a negative inotropic response. However, this negative inotropic effect proved insensitive to α-adrenoceptor antagonists. This may imply that either a physico/chemical process is involved, or that α-adrenoceptor agonists exert their negative inotropic effects after oxidative stress via other receptor/signal transduction pathways. The latter cannot be excluded since no other inotropic agent studied by us exerted a negative inotropic response and the three α-adrenoceptor agonists we have tested are chemically not related. Another explanation for this phenomenon may be that α-adrenoceptor antagonist are less effective because of changes in binding characteristics of the receptors caused by free radicals. The negative inotropic responses appear not to be the direct result of an activation of phospholipase C (PLC) or protein kinase C (PKC). Angiotensin II, which exerts a chronotropic effect via stimulation of PLC, appeared not to induce a negative intropic effect in atria exposed to free radicals. Moreover, chelerythrine was unable to counteract the negative inotropic effect of methoxamine in atria subjected to oxidative stress. Further research to analyse the underlying mechanism is ongoing.

In conclusion, free radicals clearly alter the inotropic responses to various inotropic stimuli. α-Adrenoceptor agonists exert negative inotropic effects in isolated atria after oxidative stress which are insensitive to α-adrenoceptor antagonists.

References

Baker JE, Felix CC, Olinger GN, Kalyanaraman B (1988) Myocardial ischaemia and reperfusion: direct evidence for free radical generation by electron spin resonance spectroscopy. Proc Natl Acad Sci USA 85:2786–2789

Barreto JC, Smith GS, Strobel NHP, McQuillin PA, Miller TA (1995) Terephthalic acid: a dosimeter for the detection of hydroxyl radicals in vitro. Life Sci 56:89–96

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chahine R, Chen X, Yamaguchi N, de Champlain J, Nadeau R (1991) Myocardial dysfunction and norepinephrine release in the isolated rat heart injured by electrolysis-induced free radicals. J Mol Cell Cardiol 23:279–286

Clague JR, Harvey R, Langer GA (1993) Protamine and other polycationic drugs inhibit calcium leak in cardiac cells during metabolic inhibition and free radical exposure. J Pharmacol Exp Ther 267:1349–1354

de Keulenaer GW, Andries LJ, Sys SU, Brutsaert DL (1995) Endothelin mediated positive inotropic effect induced by reactive oxygen species in isolated cardiac muscle. Circ Res 76:878–884

Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O (1985) Oxygen mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity. J Mol Cell Cardiol 17:937–945

Field ML, Azzawi A, Unitt JF, Seymour AC, Henderson C, Radda GK (1996) Intracellular calcium staircase in the isovolumic pressure-frequency relationship of Langendorff-perfused rat heart. J Mol Cell Cardiol 28:65–77

Freeman BA, Crapo JD (1982) Biology of disease: Free radicals and tissue injury. Lab Invest 47:412–426

Fridovich I (1974) Superoxide dismutases. Adv Enzymol 41:35–48

Haenen GRMM, van Dansik P, Vermeulen NPE, Timmerman H, Bast A (1988) The effect of hydrogen peroxide on beta adrenoceptor function in the heart. Free Radic Res 4:243–249

Haenen GRMM, Veerman M, Bast A (1990) Reduction of beta-adrenoceptor function by oxidative stress in the heart. Free Radic Biol Med 9:279–288

Halliwell B, Gutteridge JMC, Cross CE (1992) Free radicals, antioxidants and human disease: Where are we now? J Lab Clin Med 119:598–620

Han G, Abel PW (1987) Alfa-1-adrenoceptor subtypes linked to different mechanisms for increasing calcium in smooth muscle. Nature 325:333–335

Hoffman BF, Kelly JJ (1959) Effect of rate and rhythm on contraction of rat papillary muscle. Am J Physiol 197:1199–1204

Jackson CV, Mickelson JK, Stringer K, Rao PS, Lucchesi BR (1986) Electrolysis-induced myocardial disfunction. A novel method for the study of free radical mediated tissue injury. J Pharmacol Toxicol Methods 15:305–320

Kaneko M, Beamish RE, Dhalla NS (1989) Depression of heart sarcolemmal calcium pump activity by oxygen free radicals. Am J Physiol 256:H347–H368

Kaneko M, Chapman DC, Ganguly PK, Beamish RE, Dhalla NS (1991) Modification of cardiac adrenergic receptors by oxygen free radicals. Am J Physiol 260:H821–H826

Kukreja RC, Hess ML (1992) The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. Cardiovasc Res 26:641–655

Lucas SK, Gardner TJ, Flaherty JT, Bulkley BH, Elmer EB, Gott VL (1980) Beneficial effects of mannitol administration during reperfusion after ischaemic arrest. Circulation 62 [Suppl 1]:I-34–I-41

McCay PB (1985) Vitamin E: interactions with free radicals and ascorbate. Ann Rev Nutr 5:323–340

McCord JM (1985) Oxygen-derived free radicals in postischaemic tissue injury. N Eng J Med 312:159–163

Meerson FZ, Kagan VE, Kozlov YP, Belkina LM, Arkhipenko YV (1982) The role of lipid peroxidation in the pathogenesis of ischaemic damage and the antioxidant protection of the heart. Basic Res Cardiol 77:465–485

Meggs LG, Huang H, Li P, Capasso JM, Anversa P (1992) Chronic coronary arterial stenosis impairs alfa-1-adrenoceptor signalling and cardiac performance in rats. Am J Physiol 263:H929–H938

Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, Inoue M (1991) Does superoxide underlie the pathogenesis of hypertension? Proc Natl Acad Sci USA 88:10045–10048

Niu XL, Liu LY, Hu ML, Chen X (1995) Some similarities in vascular effects of oleic acid and oxidized low-density lipoproteins on rabbit aorta. J Mol Cell Cardiol 27:531–539

Opie LH (1989) Reperfusion injury and its pharmacologic modification. Circulation 80:1049–1063

Prasad K, Kalra J, Chan WP, Chaudhary AK (1989) Effect of oxygen free radicals on cardiovascular function at organ and cellular levels. Am Heart J 117:1196–1202

Rowe GT, Manson NH, Caplan M, Hess ML (1983) Hydrogen peroxide and hydroxyl radical mediation of activated leukocyte depression of cardiac sarcoplasmic reticulum. Circ Res 53:584–591

Tani M (1990) Mechanisms of calcium overload in reperfused ischaemic myocardium. Annu Rev Physiol 52:543–559

Tesfamariam B, Cohen RA (1992) Free radicals mediate endothelial cell disfunction caused by elevated glucose. Am J Physiol 263:H321–H326

Van den Ende R, Batink HD, Michel MC, Van Zwieten PA (1994) Influence of ischaemia and reperfusion on cardiac signal transduction. G-protein content, adenylyl cyclase activity, cyclic AMP

content and forskolin and dibutyryl cyclic AMP-induced inotropy in the rat Langendorf heart. Fundam Clin Pharmacol 8:408–416

Wang SY, Clague JR, Langer GA (1995) Increase in calcium leak channel activity by metabolic inhibition or hydrogen peroxide in rat ventricular myocytes and its inhibition by polycation. J Mol Cell Cardiol 27:211–222

Witztum JL (1994) The oxidation hypothesis of atherosclerosis. Lancet 344:793–795

Zweier JL, Flaherty JT, Weisfeldt ML (1987) Direct measurement of free radical generation following reperfusion of ischaemic myocardium. Proc Natl Acad Sci USA 84:1404–1407