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Electrical and Mechanical Modulations by Oxygen-derived Free-radical Generating Systems in Guinea-pig Heart Muscles

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Abstract

The effects of free-radical generating systems and angiotensin-converting enzyme (ACE) inhibitors on the action potentials and contractile force in guinea-pig cardiac muscles were examined using conventional microelectrode and whole-cell voltage-clamp methods at 36°C.

Hydrogen peroxide (30–100 μM) prolonged 50%, 75% and 90% repolarization of action-potential duration (APD) approximately 15–25 min after its application. But the longer exposure reversed the APD shortening in a concentration-dependent manner. Other action-potential parameters were not altered to a significant extent. The contractile force was increased. Longer exposure inhibited the enhanced force (but it was still larger than control). The effects on the spontaneous action potential from right atrial muscle were almost the same. In whole-cell voltage-clamp experiments, H_2O_2 (100 μM) inhibited L-type Ca^{2+} current and enhanced delayed rectifier K^+ current. The effects of light-activated rose bengal (10–100 nM) on the APD were similar to, but more potent than, those of H_2O_2. The response was observed rapidly after a light illumination. During exposure to rose bengal (100 nM), abnormal spontaneous action potentials or arrhythmias such as a bigeminy occurred, presumably because of early and delayed afterdepolarizations. The responses were irreversible. At 300 μM ACE inhibitors, captopril and enalapril, protected the changes induced by these free radicals.

These results indicate that H_2O_2 has a dual, time-dependent, action on the APD and rose bengal with light illumination produced the responses rapidly. The oxygen-derived free radicals increased $[Ca]_i$ and then cellular Ca^{2+} overload occurred. These responses were protected by ACE inhibitors.

Post-ischaemic stunning occurs in two phases: a first causative phase arises rapidly after onset of reperfusion and cellular Ca^{2+} overload occurs; stunning occurs as the second phase (Mercier et al 1982; du Toit & Opie 1992). The generation of cytotoxin free-radicals is observed within 1 min after reperfusion (during the first phase) (Kaplinsky et al 1981; Zweier et al 1987). During post-ischaemic reperfusion oxygen-derived free radicals can cause specific myocardial injury in addition to the damage caused by ischaemia itself. Superoxide anion ($^{\cdot}O^{-}{}_2$) generates considerable amounts of hydroxyl radicals ($^{\cdot}OH^{-}$) and has been implicated in the pathogenesis of this phenomenon (Ambrosio et al 1987; Garlick et al 1987). The responses to cytotoxic free radicals might be closely associated with specific membrane components, such as many ionic channels or exchange proteins. Thus, the free radicals alter the action potential configuration. Simultaneously the refractory period is reduced and lethal arrhythmias occur: Q-T prolongation, T wave inversion, AV block, VPC and VT (or VF) (Pallandi et al 1987; Hearse et al 1989).

It is known that free radicals are scavenged by many specific enzymes, e.g. superoxide dismutase for superoxide and catalase for superhydroxide, which can improve the recovery of cardiac function and metabolism and reduce infarct size in reperfused hearts (Burton 1985; Werns et al 1985). It has recently been shown that angiotensin converting enzyme (ACE) inhibitors also have antioxidant or scavenging action in the ischaemic and reperfused heart (Pfeffer et al 1988).

In these experiments, therefore, H_2O_2 and rose bengal photo-activated with 500–600 nm wavelength light were used to generate free radicals and the responses to these were examined. Photodynamic damages induced by light-sensitive dyes and molecular oxygen during exposure to visible light illumination have been described (Shattock et al 1991). Such responses are obtained when photo-active rose bengal is irradiated with light. We examined the electrophysiological changes (or damage) resulting from the free-radical generating systems, and also sought to examine the modulation of the action potentials and the contractile force by an application of ACE inhibitors under the pathophysiological conditions.

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Materials and Methods

Solutions

The composition of modified Tyrode solution (mM) was: NaCl 110, KCl 5·4, $MgCl_2$ 1, $CaCl_2$ 1·8, NaH_2PO_4 0·3, HEPES 5 and glucose 5. The solution was oxygenated with O_2. The pH was adjusted to 7·4 with NaOH. In whole-cell voltage-clamp experiments, the pipette (intracellular) solution contained (mM): KCl 130, Na_2ATP 5, creatine phosphate 5, EGTA 4, $MgCl_2$ 5 and HEPES 10. Intracellular pH was adjusted to 7·2. To avoid the influences of other currents, tetrodotoxin (10 μM) and 4-aminopyridine (5 mM) were added to the bath solution.

Rose bengal was obtained from Nacalai Tesque (Kyoto, Japan), captopril from Sankyo Pharmaceutical (Tokyo, Japan) and enalapril from Banyu Pharmaceutical (Tokyo, Japan).

Papillary muscle preparation experiments

Guinea-pigs, 250–350 g, were anaesthetized with pentobarbital sodium (30 mg kg^{-1}, i.p.) and the heart was quickly

excized. The papillary muscles were placed in an organ bath and superfused with Tyrode solution at 36°C, as described previously (Satoh & Vassalle 1985, 1989, 1996). The preparation was constantly driven at 1 Hz by use of a stimulator (Nihon Kohden SEN-7103, Tokyo, Japan). The stimulation voltage was approximately 50% above the threshold, and the duration was 1–2 ms. Recording of the action potentials (Nihon Kohden VC-12 and RTA-1200M) was achieved by the conventional microelectrode technique. One end of preparation was fixed to the base of the bath with a pin; the other end was connected to a force-transducer (Nihon Kohden SB-1T) by means of a short silk thread.

Single cell preparation experiments

The dissected heart was perfused with normal Tyrode solution, on a Langendorff apparatus, as previously reported (Satoh 1993). After wash-out of blood the preparations were incubated in Ca^{2+}-free Tyrode solution containing 0·1% collagenase (Type I; Sigma, St Louis, MO) for 15–20 min at 36°C. The preparations were transferred to high-K^+, low-Cl^- solution, and stored at 4°C for over 1 h.

Whole-cell patch voltage-clamp experiments were performed with an Axopatch patch-clamp amplifier (Axon Instruments, Burlingame, CA). Glass patch pipettes were fabricated with a two-stage puller. The tip diameter was 3–5 μm and the resistance was 3–5 MΩ. The data were stored and analysed on a microcomputer (NEC-PC98). Test pulses were applied once every 20 s. Current traces were filtered at a cut-off frequency of 2 kHz for plotting. All experiments were performed at 36°C.

Generation of oxidant stress

Free radicals were generated by application of H_2O_2 and by photo-activation of rose bengal, illumination of which generates oxidant stress as a result of free-radical formation (Shattock et al 1991). The decay of the rose bengal triplet state generates singlet oxygen (75%) and superoxide (20%) (Lee & Rodgers 1987). In the present experiments Tyrode solutions containing rose bengal were photo-activated by illumination of the superfusion chamber with broad-band white light of intensity approximately 2600 lux.

Statistical analysis

All data are expressed as mean ± s.e.m. Differences were analysed by Student's *t*-test and analysis of variance; $P < 0.05$ was considered as indicative of significance.

Results

Effects of H_2O_2 and rose bengal

The electrophysiological effects of H_2O_2 (10–100 μM) on action potentials and contractile force were examined. Fig. 1A shows typical changes induced by H_2O_2 (100 μM). The preparations were stimulated at 1 Hz. H_2O_2 had a dual action on the action-potential duration (APD). The increase in APD at 50, 75 and 90% re-polarization reached a peak approximately 18 min after application, then the APD decreased. The responses were both time- and concentration-dependent. The time-dependent changes in APD_{75} (75% re-polarization) and contractile force are presented in Fig. 1B. The contractile force also increased and then decreased from the peak value, but the value was still larger than the control value. The effect was not synchronous with that of APD_{75}. The data were obtained from steady-state responses approximately 30–40 min later. The changes are summarized in Table 1. After wash-out the APD_{75} shortening was recovered at 50 min later, but the contractile force was further increased.

The spontaneous action potential in the right atrium was reduced by 32·4 ± 2·5% (n = 3, $P < 0.05$) in the presence of H_2O_2 (100 μM), but the cycle length was unaffected, as shown in Fig. 2. The cycle length in three preparations was 507 ± 14 ms in control, and 501 ± 12 ms at 100 μM H_2O_2.

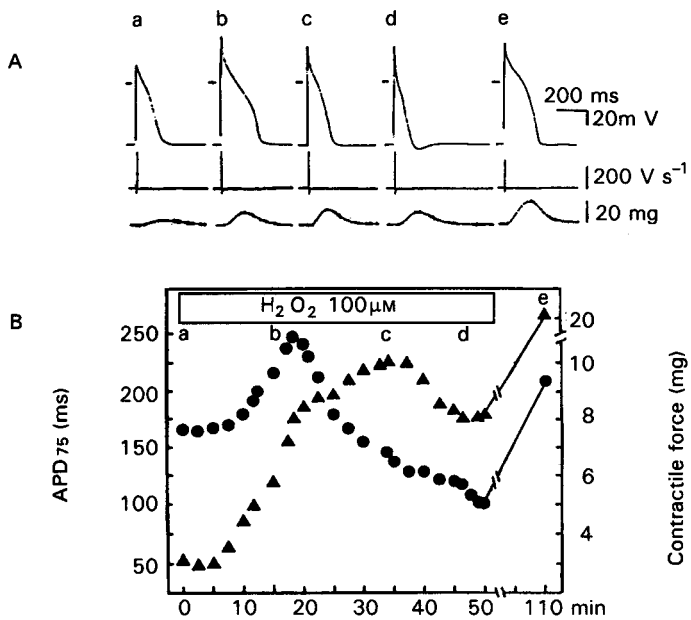

FIG. 1. Effects of H_2O_2 on action potentials and contractile force in guinea-pig ventricular muscle. The preparation was driven at 1 Hz. A. action potentials, the maximum rate of rise of depolarization and the contractile force at different times in the absence and presence of H_2O_2 (100 μM). The short line represents 0 mV. B. Time-dependent alteration of the action potential duration at 75% repolarization (●) and of the contractile force (▲).

Table 1. Effects of oxygen-derived free radicals on action potentials in guinea-pig ventricular muscle.

	Control	Hydrogen peroxide		
		10 μM	30 μM	100 μM
Action potential amplitude	124 ± 7	124 ± 9	125 ± 8	122 ± 14
Action potential duration at 75% re-polarization	157 ± 6	158 ± 7	135 ± 10*	108 ± 13**
Resting potential	−85 ± 2	−85 ± 4	−86 ± 2	−86 ± 3
Maximum rate of depolarization	253 ± 11	245 ± 12	252 ± 9	250 ± 13
Developed tension	16 ± 2	13 ± 1	10 ± 2	8 ± 3**
	Control	Rose bengal		
		10 nM	30 nM	100 nM
Action potential amplitude	124 ± 5	123 ± 9	123 ± 6	121 ± 11
Action potential duration at 75% re-polarization	155 ± 9	152 ± 6	129 ± 11*	100 ± 11**
Resting potential	−86 ± 2	−86 ± 3	−85 ± 2	−84 ± 4
Maximum rate of depolarization	267 ± 12	259 ± 10	258 ± 11	255 ± 10
Developed tension	14 ± 3	15 ± 2	11 ± 2	7 ± 3***

Values are means ± s.e.m. (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant compared with control value.

In whole-cell voltage-clamp experiments, H_2O_2 induced marked changes 7 to 10 min later (Fig. 3A). H_2O_2 (100 μM) inhibited L-type Ca^{2+} current (I_{Ca}) at +10 mV by 28·5 ± 1·4% (n = 5, $P < 0.01$), and enhanced the delayed rectifier K^+ current (I_K) at +70 mV by 16·3 ± 1·2% (n = 5,

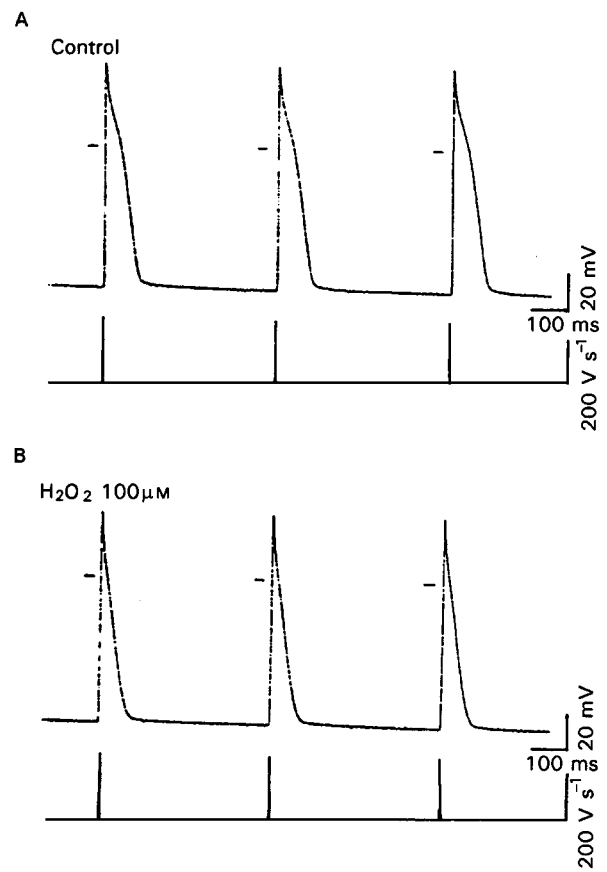

FIG. 2. Effect of H_2O_2 on the spontaneous action potentials in guinea-pig right atrium. A. Control. B. Changes in the spontaneous action potentials in the presence of H_2O_2 (100 μM); the short line represents 0 mV.

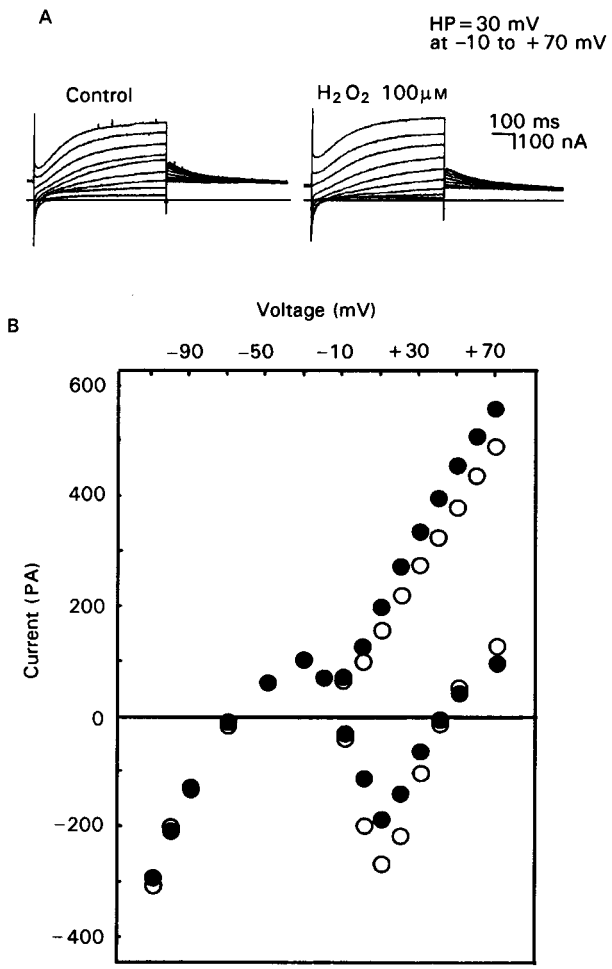

FIG. 3. Changes in ionic currents in a guinea-pig ventricular cardiomyocyte. A. Family of ionic current traces in control and H_2O_2 (100 μM). Pulses were applied between −10 to +70 mV from a holding potential of −30 mV. The pulse duration was 500 ms. The horizontal line indicates zero current level. B. Current–voltage relationship for the ionic currents. ○, Control; ●, 100 μM H_2O_2.

$P < 0.01$; Fig. 3B). The inwardly rectifying K^+ current (I_{K1}) was not modified.

Superfused solution and the preparations were pre-treated with rose bengal (10–100 nM) then irradiated with light at 2600 lux. The electrical and mechanical responses to rose bengal appeared rapidly after irradiation. Figs 4A,B show the changes in the action potentials and contractile force during exposure to rose bengal (100 nM) plus light illumination. The preparation was stimulated at 1 Hz. The effect of rose bengal on the action potentials was closely similar to that of H_2O_2, but the spontaneous or abnormal action potentials appeared between the action potentials stimulated at 1 Hz, in the form of a bigeminy. The data, obtained from steady-state responses approximately 20–30 min later, are summarized in Table 1. The resting potential (−84 to −86 mV) was unaffected. At 3 Hz the abnormal action potential was abolished, but rose bengal (100 nM) elicited early and delayed after-depolarizations (Fig. 4C). The contractile force was stimulated. These responses were not recovered completely after wash-out.

Effects of ACE inhibitors

Fig. 5A shows that H_2O_2 at 100 μM had a dual action on APD_{75}. When APD_{75} was subsequently reduced, application of

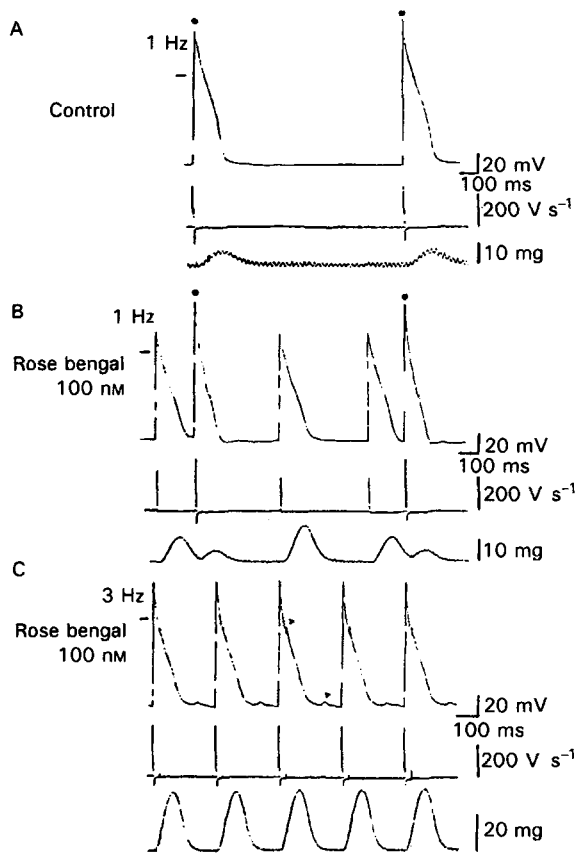

FIG. 4. Effects of rose bengal on the action potential configuration and on the contractile force. A. Control. The preparation was constantly driven at 1 Hz, indicated by the dots above the action potential recordings. B. Arrhythmias induced by rose bengal (100 nM) and light irradiation (2600 lux). The preparation was driven at 1 Hz. C. Occurrence of triggered activity in the presence of rose bengal (100 nM) and light irradiation (2600 lux). The preparation was driven at 3 Hz. Note the appearance of early and delayed after-depolarizations, indicated by arrows. The short line represents zero mV.

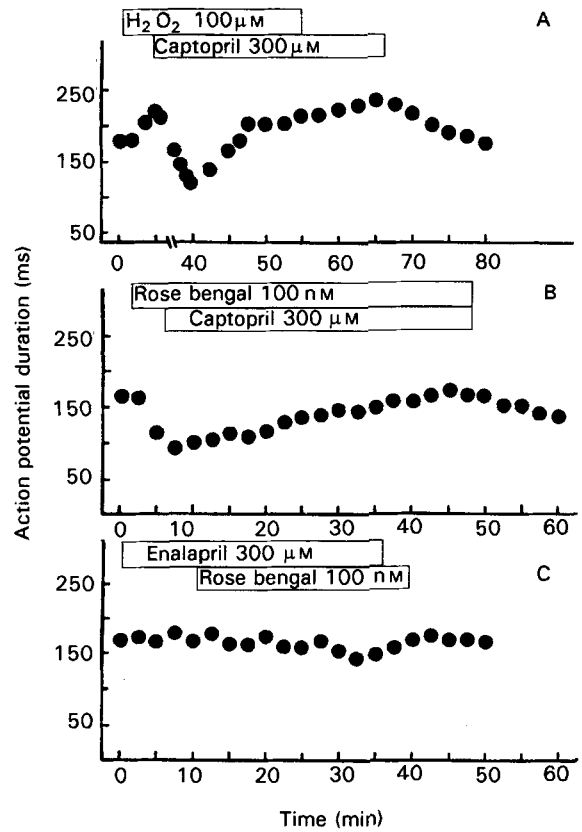

FIG. 5. Modulation of action potential duration by ACE inhibitors in the presence of free-radical-generating systems. The preparation was stimulated at 1 Hz. A. Effect of captopril (300 μM) in the presence of H_2O_2 (100 μM). B. Effect of captopril (300 μM) in the presence of rose bengal (100 nM). C. Effect of pretreatment with enalapril (300 μM).

captopril (300 μM) reversed the change induced by 40-min exposure to H_2O_2 (100 μM). The captopril-induced increase was larger than the control value. After wash-out the APD_{75} began to decrease. In the presence of rose bengal (100 nM) and irradiation with light, the shortened APD_{75} was again recovered in response to captopril (300 μM) (Fig. 5B). Fig. 5C shows that pre-treatment with enalapril (300 μM) failed to evoke any changes even in the presence of rose bengal. In these experiments with ACE inhibitors, wash-out of the drugs did not affect APD_{75}, an effect quite different from that resulting from discontinuation of treatment with H_2O_2. Similar effects were observed in 5 or 6 preparations with each procedure.

Discussion

These experiments with guinea-pig heart muscles showed that: H_2O_2 initially prolonged APD then subsequently shortened APD; photo-activated rose bengal rapidly induced arrhythmias as a result of triggered activity; the contractile force was enhanced but longer exposure inhibited the force; the responses were concentration-dependent, and the effect of rose bengal was more potent than that of H_2O_2; H_2O_2 inhibited I_{Ca} and enhanced I_K; oxygen-derived free radicals caused cellular Ca^{2+} overload; and ACE inhibitors (captopril and enalapril) prevented free-radical-induced damage. Because free radicals

did not affect other parameters of the action potentials, such mechanisms would contribute to the progressive nature of free radical-induced changes in membrane function.

Electrical effects

The plateau phase and duration of the action potentials are rapidly altered by the injury caused by free radicals. The APD increased initially and then decreased. This is consistent with results obtained from the rabbit sino-atrial (SA) node exposed to *t*-butyl hydroperoxide, a biphasic effect on I_{Ca} (Sato et al 1989). This biphasic effect would reflect changes in the plateau height of the action potentials. APD is, on the other hand, also regulated by the delayed rectifier K^+ current (I_K). Sato et al (1989) reported that free radicals reduce I_K; this was not observed in this study. Such reduction might explain the APD prolongation during the relatively early phase of exposure to H_2O_2. The I_K in this study was enhanced, leading to the subsequent APD shortening. The I_K was Ca^{2+}-sensitive, and is also known as a Ca^{2+}-activated K^+-current (I_{KCa}). Free radicals did not affect resting potential, a conclusion supported by little or no effect on I_{K1}; this result is consistent with those of Cerbai et al (1991). The initial prolongation of APD could, therefore, be a result of I_{Ca} increase and I_K decrease, and the secondary APD shortening a result of I_{Ca} decrease and I_K enhancement.

Goldhaber et al (1989) have reported that H_2O_2 caused an early efflux of K^+ from rabbit septa, and that the ATP-sensitive K^+ channel (measured as I_{KATP}) was activated by the free-radical–generating systems, H_2O_2 or xanthine + xanthine oxidase. I_{KATP} channels are activated under conditions of low ATP levels in cytosol, e.g. during ischaemia. Thus, the channels might have little or no contribution to normal action potentials, but are sensitive to the cell's metabolic state (Satoh 1993). The efflux of cellular K^+ through I_{KATP} channels might account, in part, for the reduction in APD.

Changes in $[Ca]_i$ level

Physiological increase in Ca^{2+} influx from the outside, or Ca^{2+} release from internal stores, modulates the activity of several ion channels and serves as a part of the second messengers for receptor-mediated phenomena. During exposure to free radicals, an elevation of $[Ca]_i$ level was indicated by the increase in APD and the enhancement of contractile force. Purine, xanthine oxidase and iron-loaded transferrin-generating system directly increased $[Ca]_i$ in cultured neonatal rat cells treated with fura-2 (Burton et al 1990). The effects on the APD and contractile force were not simple, however, but were biphasic with time. The subsequent changes might indicate an extreme increase in $[Ca]_i$ level, i.e. occurrence of Ca^{2+} overload. The decrease in the APD and the depression of tension as the second actions of H_2O_2 are consistent with the results by Barrington et al (1988) as are the abnormal action potentials and triggered activity (early and delayed after-depolarizations) in experiments with rose bengal. The force, furthermore, was enhanced at 3 Hz. Under normal conditions increasing frequency reduces the force (Satoh & Vassalle 1985, 1996).

The triggered activity is elicited by oscillatory Ca^{2+} release from sarcoplasmic reticulum (SR). The oscillatory potentials are responsible for a transient inward current (I_{ti}), abolished by caffeine, and for an inward tail current (I_{ex}) (Hasegawa et al 1987; Satoh & Vassalle 1989; Satoh et al 1989). Thus I_{ti} would be elicited by Na/Ca exchange current or Ca^{2+}-activated non-selective current (Kass et al 1978; Lee et al 1985). The oscillations in membrane potentials and developed tension are likely to underlie the arrhythmias in isolated hearts exposed to oxidant stress. The mechanisms might include increased sarcolemmal permeability to Ca^{2+} ions, an effect on I_{Ca} inactivation, mitochondrial impairment, and damage to the Ca^{2+} release channel of SR (Holmberg et al 1991). Free radicals would reduce the removal of $[Ca]_i$ through Ca^{2+}-ATPase activity in the SR and sarcolemma (Mak et al 1985). Free radicals can, in addition, hinder the activity of the Na^+ pump and deinhibit Na/Ca exchange (Reeves et al 1986; Kukreja et al 1991). In the current experiments, therefore, wash-out of H_2O_2 resulted in recovery of the depressed contractile force owing to release from depressant SR functions (Satoh & Vassalle 1985, 1989, 1996). These results indicate, therefore, that the $[Ca]_i$ increase developed slowly and excess cytosolic Ca^{2+} level (Ca^{2+} overload) occurred during the relatively longer exposure to free radicals generated by H_2O_2 and by rose bengal after light illumination.

Protection by ACE inhibitors

Free radical-induced damage during ischaemia–reperfusion can be reduced by reducing the generation of free radicals (e.g. by administration of allopurinol and catalase) or by scavenging of free radicals (e.g. by administration of ascorbic acid, α-tocopherol, superoxide dismutase or mannitol) (Pallandi et al 1987). ACE inhibitors might be of benefit in the acute phase of myocardial infarction. The apparently salutary effects of ACE inhibitors on myocardial infarct size, coronary blood flow, systemic haemodynamics, neuroendocrine activation, thrombolytic therapy, and reperfusion injury have already been shown in experimental models (Ambrosioni & Borghi 1989) and in clinical applications (Pfeffer et al 1988).

In the current experiments ACE inhibitors prevented the responses induced by free radicals. ACE inhibitors scavenge hydroxyl radicals more strongly than the superoxide anion radical. In the presence of ACE inhibitors, removal of the free radicals resulted in no increase in APD_{75}. This is quite inconsistent with the results of wash-out of H_2O_2 in the absence of ACE inhibitors (see Fig. 1B). This indicates that ACE inhibitors prevent the occurrence of Ca^{2+} overload. Captopril (a prototype containing –SH groups) inhibits hydroxyl radicals in a concentration-dependent manner, whereas enalapril (containing no –SH groups) is inert (McMurray & Chopra 1991). However, enalapril has also been found to be involved in the modulation of cell communication (improvement of electrical synchronization) in cardiac muscles and to have beneficial effects in patients with congestive heart failure (De Mello & Altieri 1991). In the current experiments both ACE inhibitors had protective action, although relatively higher concentrations were used. The protective concentration of ACE inhibitors is 20–100 μM in in-vitro experiments, and 3–5 (or 3–10) mg kg^{-1} in in-vivo experiments (McMurray & Chopra 1991). Because the protocols employed to generate free radicals in in-vitro experiments are quite different from those in in-vivo experiments (i.e. during reperfusion), and because the drug concentrations are not measured in blood or tissues, no attempt was made to explain the different concentrations effective in-vitro and in-vivo.

The number of binding sites for nitrendipine (Ca^{2+} antagonist) was reduced in membrane vesicles exposed to free radical-generating systems, such as xanthine plus xanthine oxidase, H_2O_2 or H_2O_2 plus Fe^{2+} (Kaneko et al 1989). The Ca^{2+} channel might be a site for free-radical injury. Other scavengers also have pronounced protective action against ischaemia–reperfusion damage, as a result of suppression of development of oxidative stress and free radicals (Ferrari et al 1989; Massey & Burton 1990). Further experiments are required to elucidate the mechanisms in detail, but the information could help clarify in-vivo mechanisms for free radical generation and damage during pathological insult.

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