The antioxidant effects of a novel iron chelator salicylaldehyde isonicotinoyl hydrazone in the prevention of H_2O_2 injury in adult cardiomyocytes

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Abstract

Objective: This study was designed to investigate the cardioprotective effect of the novel lipophilic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) against the oxidative stress exerted by H_2O_2 through the production of $^{\cdot}OH$ radical via the Fenton reaction and to compare them with those of the hydrophilic iron chelator deferoxamine (DFO) and the Na^+/H^+ exchange inhibitor methylisobutyl amiloride (MIA). **Methods:** We used long-term cultures of spontaneously beating adult guinea-pig ventricular cardiomyocytes developed and characterized previously in our laboratory. We assessed their contractile activity by video-recording as well as the underlying Ca_i^{2+} transients by Fura 2 fluorescence. In some experiments we also recorded these functional parameters, plus the electrical activity (action potentials) in response to electrical stimulation via suction pipettes, in individual freshly isolated myocytes. **Results:** Exposure of the regularly and synchronously beating cultured cardiomyocytes to 100 μM H_2O_2 initially caused a substantial prolongation of Ca_i^{2+} transients accompanied by an irregular contractile activity, then in contractile arrest and a severalfold increase in cytosolic $[Ca^{2+}]$ that occurred, within 30 min of H_2O_2 application. Similar effects were also observed using freshly isolated cardiomyocytes. The latter effects were first accompanied by significant prolongation of the action potential duration (APD) with superimposed early afterdepolarizations followed by a second phase with a very fast decrease in APD, contractions, as well as Ca_i^{2+} transients and a third phase of inexcitability, contractile arrest, increased cytoplasmic $[Ca^{2+}]$ and a final contracture. All these effects were irreversible in both types of preparations but they could be fully prevented by a 15-min preincubation with 200 μM SIH. Similar protective effects were observed with DFO, but in this case a much higher concentration had to be used (1 mM) and much longer (2 h) preincubation was needed. By contrast, 5 μM MIA failed to fully protect the cardiomyocytes, although a significant delay (10 min) of the effects of H_2O_2 was observed. **Conclusions:** The data indicate that SIH provides a very powerful and very fast protection against the oxidative stress exerted by H_2O_2 presumably via the iron-mediated Fenton reaction producing hydroxyl radical ($^{\cdot}OH$), whereas the protective effect of DFO is hindred by its very slow and rather limited intracellular entry, and the protection that MIA exerts via the inhibition of Na^+/H^+ exchange against H_2O_2 much less effective. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

It has been proposed that oxygen-derived oxidants, such as the superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), could be important mediators of arrhythmias that occur during reperfusion of the ischemic myocardium [1]. More recently, electron paramagnetic resonance and histochemical studies have convincingly demonstrated that these oxidants are generated early during myocardial reperfusion [2–6]. The formation of these free radicals in biological systems is catalyzed by iron which is normally non-reactive because it is bound to proteins of iron transport (transferrin) and

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storage (ferritin). Under certain abnormal conditions, such as during reperfusion injury, activated oxygen species release iron from these proteins, and the resulting "free" iron (Fe^{2+}) promotes the formation of the devastatingly reactive toxic ($\cdot OH$).

Iron-chelating agents, such as deferoxamine (DFO) have been shown to inhibit free-radical formation and the consequent free radical tissue damage in some experimental systems [7].

However, DFO crosses cell membranes inefficiently, and this feature may limit its effectiveness in vivo [8]. On the other hand, a lipophilic iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH), which enters cells and tissues very efficiently [9–11], is a powerful inhibitor of iron-dependent production of hydroxyl radical ($\cdot OH$) from H_2O_2 [12,13].

Previously [14] we studied in considerable detail the development of H_2O_2-induced changes in membrane potentials, membrane currents, and corresponding contractile activity (shortening) in rat and guinea-pig ventricular myocytes, using the suction-pipette whole-cell clamp method.

The observed changes in response to 30–100 μM H_2O_2 occurred in three phases: a prolongation of action potential duration (APD) accompanied by increased contractility, followed by a period of decreased APD and reduced contractility, with a final phase of inexcitability and the myocytes' contracture.

DFO prevented those changes at much lower concentrations and after much shorter preincubation exposures when applied intracellularly (via a suction electrode) than when applied extracellularly. This indicates that the changes were induced by $\cdot OH$ generated intracellulary in the presence of iron.

In this study, we have compared the protective effects against oxidative injury by H_2O_2 that externally applied SIH exerts with those of DFO and with those of the Na^+/H^+ exchange inhibitor methylisobutyl amiloride (MIA), which has been indicated as providing protection against some effects of H_2O_2 [15] and against superfusion injury [16,17]. We have used our model of cultured adult guinea-pig cardiomyocytes as well as in some experiments we also used freshly isolated cardiomyocytes [18–20] to determine how H_2O_2 in the presence or absence of the above drugs affects contractile activity, electrical activity, and Ca^{2+}_i transients. Our results demonstrate that SIH exerts a very fast and potent cardioprotective effect against oxidative stress inflicted by H_2O_2, which indicates the potential therapeutic significance of this lipophilic iron chelator against cardiac ischemia/reperfusion.

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Solutions and chemicals

The Ca^{2+}-free standard HEPES-buffered Tyrode's solution contained 120.5 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose, and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. This solution is referred to hereafter as Ca^{2+}-free Tyrode's solution.

Salicylaldehyde isonicotinoyl hydrazone (SIH, i.e. 2-hydroxybenzal isonicotinoyl hydrazone) was synthesized by Schiff base condensation between 2-hydroxybenzaldehyde and isonicotinic acid hydrazide, as described previously [10]. The chemical structure is given in Fig. 1.

2.2. Cardiac myocyte preparations

Details of the isolation of adult guinea-pig cardiomyocytes have been presented elsewhere [18,19]. Briefly, the aorta of anesthetized guinea pigs (300–350 g) was cannulated and perfused retrogradely for 5 min at 35°C with Ca^{2+}-free Tyrode's solution. To this solution was then added 0.16–0.18% collagenase (Worthington type II, Freehold, NJ), 0.004% trypsin (Sigma Chemical Co., St. Louis, MO), 0.4% type F (fatty acid free) albumin, and 25 μM $CaCl_2$. The concentration of collagenase was chosen to yield an optimal dissociation process. This perfusate was recirculated for 3–5 min, following which the ventricles were removed and sliced into small strips which were incubated in a shaker bath for several 15-min periods. After gentle centrifugation, cells were pooled and this cell suspension was used for plating after the cells were counted in a special 1-ml counting chamber (Sedgewick rafter; Graticules Ltd.; Turnbridge Wells, UK). At least 98% of the cells in a preparation are myocytes ($\sim$8–12×10^6 cells/animal), of which >80% are rod

Salicylaldehyde Isonicotinoyl Hydrazone (SIH)
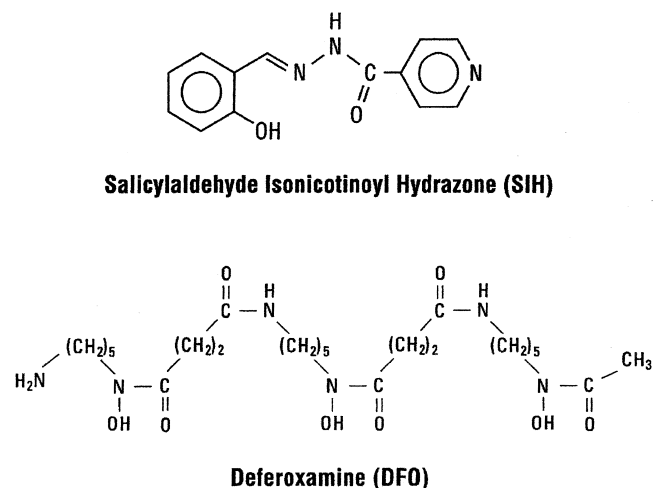
Deferoxamine (DFO)

Fig. 1. The chemical structure of SIH and DFO.

shaped and >90% are viable (as demonstrated by exclusion of Trypan Blue).

2.3. Culture techniques

Cells were cultured in Eagle's minimum essential medium with Earle's salts, supplemented as described previously [19]. Cytosine 1-β-D arabinofuranoside (10 μM) was added to the culture medium to minimize the growth of fibroblasts, and laminin (5 μg/cm) was used to coat the 1.2-cm-diameter glass coverslips. We used a plating density (10^5 myocytes/cm^2) which we established in a previous study [18] to be optimal for the culturing of guinea-pig myocytes.

Plated cells were maintained in an incubator at 37°C under a 95% air–5% CO_2 atmosphere, and culture media were replaced twice a week. Cultures used in these experiments were 3–4 weeks old, as they are at this stage fully interconnected and very stable in their synschronized beating rates [20].

2.4. Recording of contractile activity in myocytes

The details of this technique have been described previously [19]. Briefly, 1.2-cm-diameter glass coverslips with cultured myocytes were placed in a 0.5-ml bath and perfused at 3 ml/min at 35±1°C. The contractile activity (shortening) of the myocytes was recorded by a video edge-detecting system. The recording was obtained by recording the movement of the end of the isolated cell or an intercellular boundary (edge) between two or more cultured cells which represented the synchronised contractile activity of the whole confluent layer of each culture.

2.5. Measurements of [Ca^{2+}]$_i$

Recording of the Ca$_i^{2+}$ transients were performed using Fura 2-AM methodology [21]. Cultured (or freshly isolated) myocytes were loaded with 4 μM Fura 2-PE3-AM (Teflabs Inc., Austin, TX) for 60 min at room temperature under an O_2 atmosphere. The myocytes were then superfused with control Tyrode's solution containing 2.5 mM $CaCl_2$ for 30 min at 37°C on the microscope stage. The Ca$_i^{2+}$ transients were measured using a PTI-system (South Brunswick, NJ) that measures fluorescence at wavelengths of 340 and 380 nm. The Ca$_i^{2+}$ transients are expressed as the fluorescent ratio 340/380.

2.6. Recording of the electrical activity

The freshly isolated myocytes were placed into a small (0.5 ml) experimental chamber, superfused (3 ml/min) with Tyrode's solution (pH 7.4; 35±1.0°C as recorded continuously by a thermoprobe in the chamber), and gassed with 100% O_2. The concentration of $CaCl_2$ was 2.5 mM. The membrane resting and action potentials were recorded with patch pipettes where R_{el}=5–10 MΩ. The pipettes were fabricated with a Kopf (two-stage) vertical puller. An Ag–AgCl bridge connected the pipettes to the input stage of an amplifier (Axoclamp-Axon Instruments, Burlingame, CA, USA). The myocytes were intracellularly stimulated through the recording patch pipette by current pulses that were 20 ms in duration and 1–5 nA in amplitude at a frequency of 0.2 Hz or 0.5 Hz. The action potentials and the corresponding shortenings were recorded by means of pClamp software on a PC-AT 386 computer. Statistical comparison of some data has been made using Student's *t*-test for pair data which are presented as means±S.E.M.

3. Results

In this study we used regularly spontaneously beating cultured cardiomyocytes for most experiments because they are a more stable and more reproducible experimental model for recording contractile activity and Ca$_i$ transients than are freshly isolated cardiomyocytes.

However, since the electrical activity could not be directly recorded for prolonged periods of time in the spontaneously beating cultures, we also used isolated myocytes in some experiments to assess the similarity of the waveform of the Ca$_i^{2+}$ transients and the configuration of the action potential in the presence of H_2O_2. The frequency of the contractions and of the Ca$_i^{2+}$ transients within each individual culture were identical, the single regular contractions being accompanied by single Ca$_i^{2+}$ transients of approximately 500 ms duration (Fig. 2A). Within 10 min of the application of 100 μM H_2O_2 the Ca$_i$ transients became transiently largely prolonged (to about 2 s) with multiple peaks, and their diastolic baseline was slightly increased, indicating elevated resting cytosolic [Ca^{2+}] (Fig. 2B). These dramatically prolonged Ca^{2+} transients were accompanied by irregular contractile activity (Fig. 2B). During the following 10–15 min, the amplitude of the individual contractions decreased while the amplitude of the Ca^{2+} transients was not altered much, despite the fact that, the level of cytoplasmic [Ca^{2+}] during diastole further increased and they became shorter and more frequent (Fig. 2C). Within the next 10 min (i.e., 25–30 min after H_2O_2 was applied), the cultures stopped contracting and the Ca$_i^{2+}$ transients disappeared while the cystolic [Ca^{2+}] increased further (Fig. 2D), reaching a value of 2–3 times the initial one. This state was fully irreversible, and spontaneous activity did not return even after 120 min of perfusion with control Tyrode's solution (not shown).

H_2O_2 had similar effects in the freshly isolated myocytes. The contractile activity was greatly prolonged as a result of an increase of the action potential duration
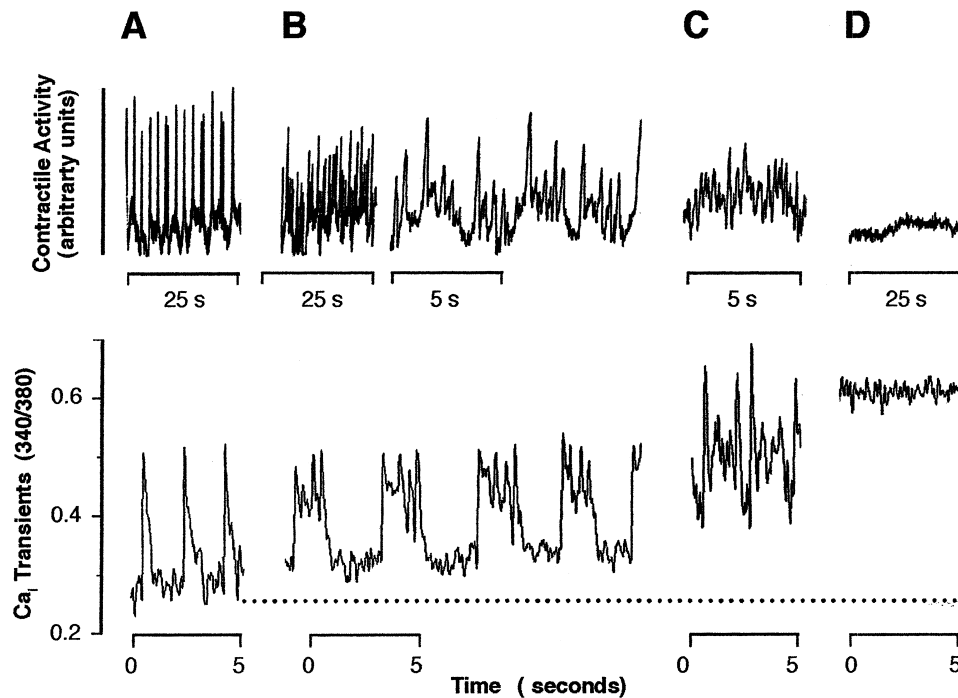
Fig. 2. The contractile activity (upper traces) and Ca_i^{2+} transients (lower traces) of 25-day-old spontaneously beating cultured adult guinea-pig cardiomyocytes in Tyrode's solution (A) and in the presence of 100 μM H_2O_2, 10 min (B), 20 min (C), and 30 min (D). Note that the baseline of Ca_i^{2+} (the control diastolic level is represented by the dotted line) increased steadily with the length of exposure to H_2O_2. Similar results were recorded in another 8 preparations.

(APD), which reached a maximum of several seconds with early afterdepolarizations (EADs) within 20 min of the application of 100 μM H_2O_2 (Fig. 3 B and C). This APD prolongation was accompanied with considerably prolonged Ca_i^{2+} transients. This effect lasted only a few minutes, while the APD varied from beat to beat between 1 and 3 s (e.g. compare Fig. 3B and C). About 25 min after the application of H_2O_2 the APD started to shorten at a very fast rate (Fig. 3D–F), and within 30–35 min after the application, the cells became inexcitable, and the contractions and Ca_i^{2+} transients subsided while the cytosolic $[Ca^{2+}]$ was increased two- to threefold (Fig. 3G). Shortly after that, the cell underwent a contracture.

All the changes in the contractile activity of the cultured myocytes and the Ca_i^{2+} transients observed in the presence of 100 μM H_2O_2 were completely suppressed when the cultures were preincubated with 200 μM SIH for 15 min prior to H_2O_2 application (Fig. 4B). In order to assure that the effect of SIH did nor represent merely a delayed process of that observed in its absence, we washed out both drugs after 40-min exposure to H_2O_2 and continued perfusion in control Tyrode's solution for another 20–30 min. Both the contractile activity and the Ca_i^{2+} transients maintained a regular frequency and pattern (Fig. 4C). 200 μM SIH itself did not affect the electrical or contractile activity or the Ca_i^{2+} transients in freshly isolated myocytes

(e.g. compare Fig. 5A and B) and it fully exerted its protective effect against 100 μM H_2O_2 up to 50 min — the longest perfusion examined (Fig. 5C–E). A small increase in the amplitude of the contractile activity was occasionally observed (Fig. 5E). However, this occurred without any significant change in APD which was: 290±13 msec and 303±16 ms for control and experimental group, respectively ($n=4$; $P>0.05$).

The hydrophilic iron chelator DFO also had protective effects against H_2O_2, but only at 1 mM, i.e., a five times higher concentration than SIH. In addition, at least 2 h preincubation with 1 mM DFO was needed to prevent (Fig. 6B) the effects of 100 μM H_2O_2 observed in its absence. There was usually a slight decrease in the frequency of spontaneous beating in the presence of DFO, which was not analyzed in detail, since it was fully reversible upon a subsequent 20-min washout in Tyrode's solution (Fig. 6C).

Finally, there was a little or no protective effect of 5 μM MIA against H_2O_2 injury in cultured myocytes. The pattern of the effects of 100 μM H_2O_2 on the contractile activity and the Ca_i^{2+} transients was similar in both the presence and the absence of MIA, although they developed more slowly (Fig. 7B–D), with the final stage of irreversible contractile arrest (Fig. 7D) occurring 15–20 min later when MIA was present ($n=6$) than in its absence.
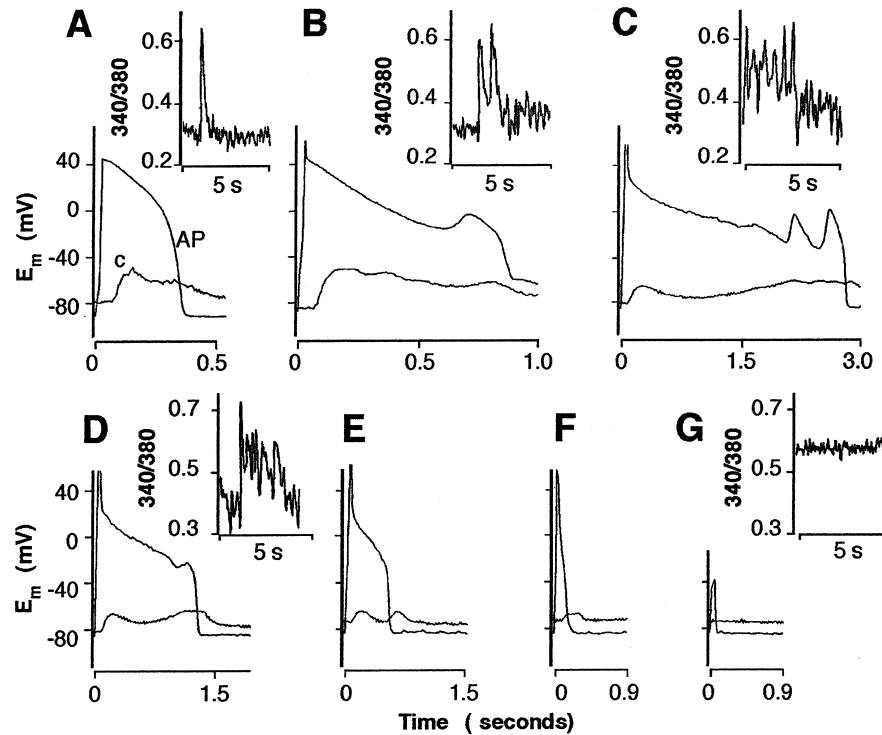
Fig. 3. The action potentials (AP) and the corresponding contractile shortening (c) and Ca_i^{2+} transients (inserts) in freshly isolated guinea-pig myocyte recorded upon stimulation at 0.2 Hz in Tyrode's solution (A), and after100 μM H_2O_2 had been applied for 15 min (B), 20 min (C), 25 min (D), 27 min (E) and 30–33 min (F and G). Similar results were obtained in other 4 myocytes. Note the different time scales in the individual recordings and the early afterdepolarizations (EADs) at the end of the AP (C).
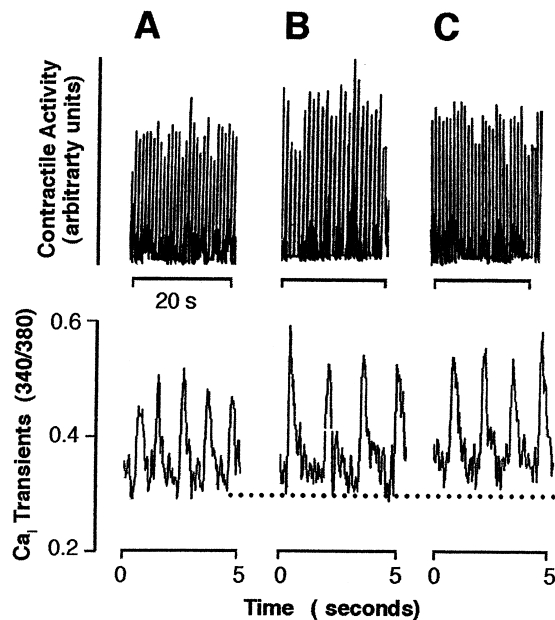

Fig. 4. The spontaneous contractile activity (upper traces) and Ca_i^{2+} transients (lower traces) of 24-day-old cultured adult guinea-pig cardiomyocytes. The culture has been preincubated for 15 min in 200 μM SIH (A) before an application of 100 μM H_2O_2 for 40 min (B). This exposure was followed by a 20-min washout in control Tyrode's solution (C). Similar results were obtained in another 7 preparations.

4. Discussion

In the present study we have demonstrated that the application of H_2O_2 leads to disturbances in spontaneous contractile activity in cultured myocytes as a result of prolonged Ca_i^{2+} transients, which in turn seems to be a result of a greatly prolonged APD (as observed in freshly isolated myocytes). Furthermore, the increased Ca^{2+} influx leads to manifold increase in diastolic concentration of cytosolic [Ca^{2+}] as demonstrated by the increase of the baseline of the Fura-2 fluorescence ratio. This Ca^{2+} overload is presumably — at least partially — responsible for the final irreversible inexitability and contractile arrest in the cultured cardiomyocytes and the contracture in the freshly isolated myocytes. All these effects were fully prevented by the lipophilic iron-chelator SIH in both the cultured and freshly isolated myocytes.

Recently, SIH was shown to prevent ˙OH-mediated (formed from Fe(III) EDTA plus ascorbate) release of TBARS from 2-deoxyribose as well as the release of ethylene from 2-keto-4-methiobutyric acid [13] and to prevent plasmid pVC-17 DNA strand breaks induced by ˙OH radicals [22]. More recently, when electron paramagnetic resonance (EPR) with 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin trap for ˙OH, SIH
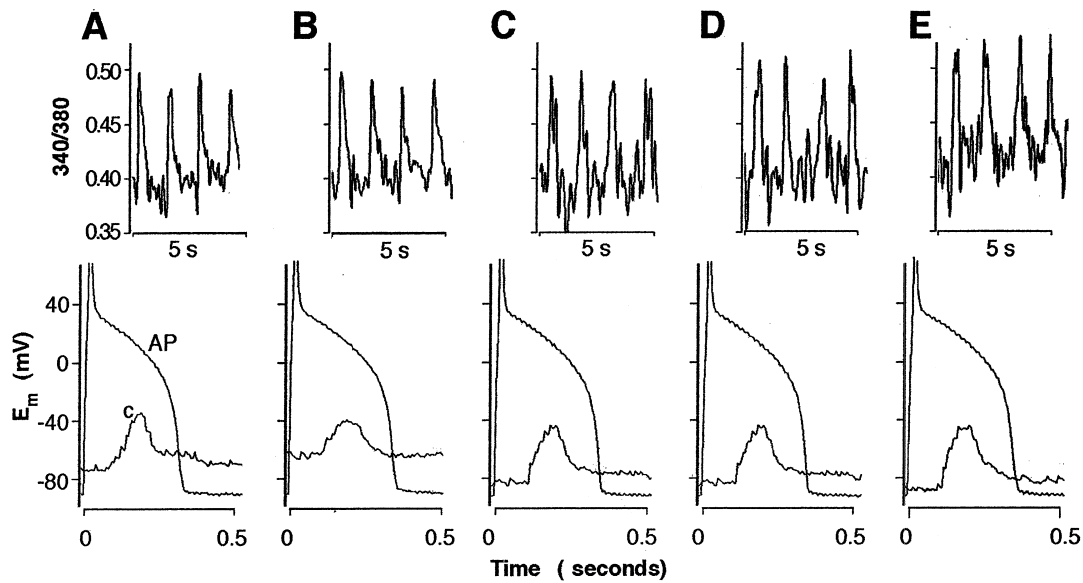
Fig. 5. The action potentials (AP), the corresponding contractile shortenings (c), and the Ca_i^{2+} transients (upper panels) recorded in freshly isolated guinea-pig cardiomyocytes upon stimulation at 0.5 Hz in Tyrode's solution (A), 15 min after the application of 200 μM SIH (B), and after 100 μM H_2O_2 has been applied in the presence of 200 μM SIH for 20 min (C), 35 min (D) and 50 min (E). Similar results were obtained in another 3 myocytes.

was shown to inhibit Fe(II)-dependent production of ˙OH from H_2O_2 [12]. Collectively, these studies indicated that SIH is a powerful inhibitor of iron-mediated oxyradical formation in vitro. The present study, indicates that SIH may also be a strong inhibitor of free-radical formation in functionally intact cells, since it can prevent electrophysio-

Fig. 6. The contractile activity (upper traces) and Ca_i^{2+} transients (lower traces) in 26-day-old spontaneously beating cultured adult guinea-pig cardiomyocytes preincubated for 2 h in 1 mM DFO in Tyrode's solution (A), after application of 100 μM H_2O_2 (in the presence of DFO) for 40 min (B) and after washout for 20 min in control Tyrode's solution (C). Similar results were obtained in another 3 preparations.

logical alterations of cardiomyocytes oxidatively damaged following their exposure to H_2O_2.

Although another chelator — DFO — was reported to be also cardioprotective against oxidative stress during ischemia/reperfusion [23,24], it was significantly less potent than SIH in preventing H_2O_2-induced electrophysiological changes in cultured cardiomyocytes (present study). This can be explained by the fact that SIH crosses cell membranes more efficiently than DFO does [10] and thus can effectively chelate intracellular iron and prevent its participation in the Fenton reaction. Indeed, a recent study by Cable and Lloyd [8] provided evidence that DFO is incapable of efficiently crossing membranes, entering cells only by endocytosis, and that it accumulates mainly in the endosome-lysosome complex. Also in concord with the present results are our earlier data [14] demonstrating that the oxidative injury of freshly isolated rat and guinea-pig cardiomyocytes by H_2O_2 was prevented immediately following the application of DFO intracellulary — via a suction pipette —, while several hour's preincubation with a much higher DFO concentration was needed when the chelator was applied extracellularly.

Since in the present study we have demonstrated that Ca^{2+} overload develops as a result of the oxidative injury caused by H_2O_2, we have investigated the possible contribution to the Ca^{2+} loading due to the activation of Na^+/H^+ exchange leading to increased $[Na^+]_i$. Such an increase in $[Na^+]_i$ could possibly subsequently induce a decrease of Na^+-dependent Ca^{2+} efflux during diastole and/or an increased Na^+-dependent Ca^{2+} influx during systole via Na^+/Ca^{2+} exchange.

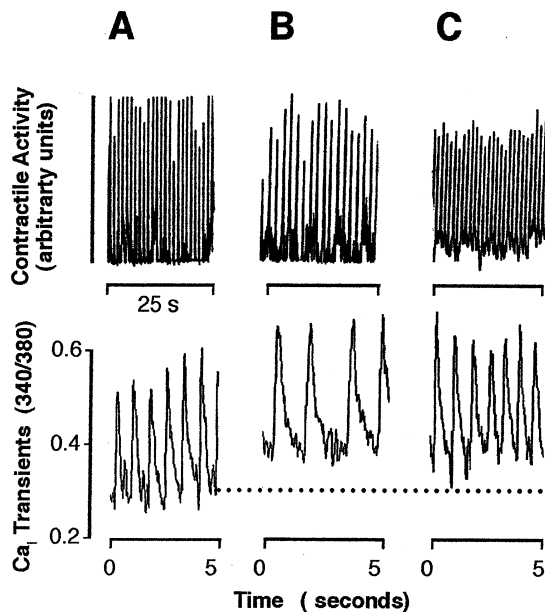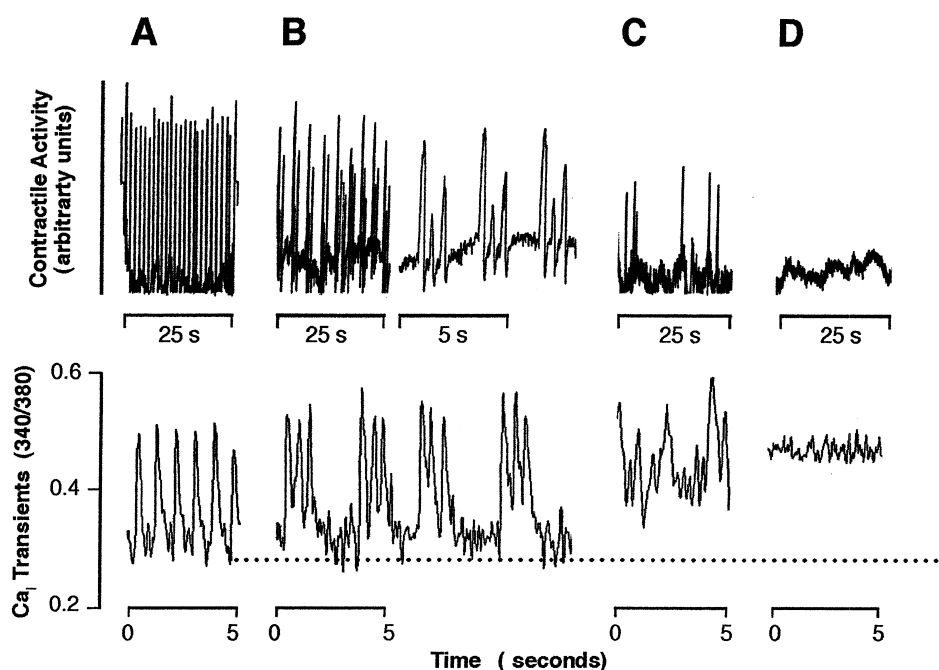Our data indicate that although blocking the Na^+/H^+ exchange by the highly potent and selective inhibitor 5 μM

Fig. 7. The contractile activity (upper traces) and Ca_i transients (lower traces) in 25-day-old spontaneously beating cultured adult guinea-pig cardiomyocytes after 30 min of preincubation with 5 μM MIA (A) and upon a subsequent additional application of 100 μM H_2O_2 for 15 min (B), 30 min (C), and 45 min (D). Note the increasing diastolic level of $[Ca]_i$ in C and D; the dotted line represents the basal level of $[Ca]_i$ in the absence of H_2O_2. Similar results were obtained in another 5 experiments.

MIA [16,17] did significantly slow down the process of the oxidative injury by H_2O_2, it did not prevent it. These data indicate that some of the Ca^{2+} load may be related to the Na^+ influx via Na^+/H^+ exchange, pointing out the subsequent role of the Na^+/Ca^{2+} exchanger.

However, it is likely that most of the Ca^{2+} overload is a direct consequence of largely prolonged Na^+ influx via the Na^+ channels that results in Ca^{2+} accumulation via Na^+-Ca^{2+} exchange, as indicated by our earlier study [14]. We have demonstrated that both the increased APD and the contractile force induced by H_2O_2 could be fully blocked by tetrodotoxin. The secondary effect of the H_2O_2 producing a fast shortening of APD may be due to activation of K_{ATP} channels as suggested by Tokube et al. [25].

Thus, our results demonstrate a highly protective capacity of SIH against free-radical-mediated tissue damage and strongly suggest that this chelator may be useful in the treatment of iron-mediated oxidative stress under various pathological situations in the heart such as ischemia/ reperfusion. Hence, SIH deserves further investigation in more complex experimental models in vivo.

Acknowledgements

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