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Reactive oxygen metabolites increase mitochondrial calcium in endothelial cells: implication of the Ca^{2+}/Na^{+} exchanger

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SUMMARY

In endothelial cells, a bolus of hydrogen peroxide (H_2O_2) or oxygen metabolites generated by hypoxanthine-xanthine oxidase (HX-XO) increased the mitochondrial calcium concentration $[Ca^{2+}]_m$. Both agents caused a biphasic increase in $[Ca^{2+}]_m$ which was preceded by a rise in cytosolic free calcium concentration $[Ca^{2+}]_c$ (18 and 6 seconds for H_2O_2 and HX-XO, respectively). The peak and plateau elevations of $[Ca^{2+}]$ were consistently higher in the mitochondrial matrix than in the cytosol. In Ca^{2+}-free/EGTA medium, the plateau phase of elevated $[Ca^{2+}]$ evoked by H_2O_2 due to capacitative Ca^{2+} influx was abolished in the cytosol, but was maintained in the mitochondria. In contrast to H_2O_2 and HX-XO, ATP which binds the P2Y purinoceptors induced an increase in $[Ca^{2+}]_m$ that was similar to that of $[Ca^{2+}]_c$. When cells were first stimulated with inositol 1,4,5-trisphosphate-generating agonists or the Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA), subsequent addition of H_2O_2 did not affect $[Ca^{2+}]_c$, but still caused an elevation of $[Ca^{2+}]_m$. Moreover, the specific inhibitor of the mitochondrial Ca^{2+}/Na^{+} exchanger, 7-chloro-3,5-dihydro-5-phenyl-1H-4.1-benzothiazepine-2-on (CGP37157), did not potentiate the effects of H_2O_2 and HX-XO on $[Ca^{2+}]_m$, while causing a marked increase in the peak $[Ca^{2+}]_m$ and a significant attenuation of the rate of $[Ca^{2+}]_m$ efflux upon addition of histamine or CPA. In permeabilized cells, H_2O_2 mimicked the effects of CGP37157 causing an increase in the basal level of matrix free Ca^{2+} and decreased efflux. Dissipation of the electrochemical proton gradient by carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), and blocade of the Ca^{2+} uptake by ruthenium red prevented $[Ca^{2+}]_m$ increases evoked by H_2O_2. These results demonstrate that the H_2O_2-induced elevation in $[Ca^{2+}]_m$ results from a transfer of Ca^{2+} secondary to increased $[Ca^{2+}]_c$, and an inhibition of the Ca^{2+}/Na^{+} electroneutral exchanger of the mitochondria.

Key words: Hydrogen peroxide, Xanthine oxidase, Mitochondrial transmembrane potential

INTRODUCTION

Oxidants are produced continuously within the cells as a by-product of aerobic metabolism, with mitochondria being the major source of generation. These oxidants include superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals. They readily react with cellular macromolecules such as DNA, proteins and lipids, either damaging them directly or setting in motion a chain reaction. This results in extensive damage to cellular structures and, eventually, cell death. Recently, attention has been focused on mitochondrial Ca^{2+} homeostasis during oxidative stress, since reactive oxygen species seem to be involved in some models of programmed cell death associated with mitochondrial dysfunction (Richter et al., 1996; Petit et al., 1996). Mitochondria take up and release Ca^{2+} via separate pathways (for a review see Gunter and Pfeiffer, 1990; Gunter et al., 1994). Ca^{2+} enters the organelle via a ruthenium red sensitive uniporter driven by the membrane potential $\Delta\psi_m$ generated by the electron transport chain. Under normal cellular conditions, the mitochondrial Ca^{2+} release pathways involve antiporters that allow Ca^{2+} efflux in exchange for Na^{+} or H^{+}, the relative contribution of Na^{+} versus H^{+} to Ca^{2+} efflux depending on the source of the mitochondria. On the other hand, there exists a proteinaceous pore, a so-called megachannel, within the inner mitochondrial membrane that is normally closed but which can be opened under some pathophysiological conditions. In its open configuration, the pore allows release of Ca^{2+} from the matrix and permeation of small solutes, leading to swelling of mitochondria and loss of the transmembrane potential. Opening of the pore, referred to as the permeability transition, is regulated by matrix Ca^{2+}, but is also enhanced by oxidants and inorganic phosphate (Gunter and Pfeiffer, 1990; Zoratti and Szabò, 1995). Meanwhile, evidence is accumulating that under conditions of oxidative stress, mitochondria rapidly lose their Ca^{2+} pool (Vlessis and Mela-Riker, 1989; Fagian et al., 1990; Richter and Kass, 1991; Schlegel et al., 1992) and $\Delta\psi_m$ (Fagian et al., 1990;

Schlegel et al., 1992; Nieminen et al., 1995). This is followed by a depletion of cellular ATP content and a loss of cell viability. The oxidant-induced Ca^{2+} release from mitochondria appears to result from activation of the permeability transition and can be reversed by cyclosporin A, an immunosuppressive agent that blocks the megachannel (Crompton et al., 1988; Kass et al., 1992). Most of these studies have relied essentially upon the use of isolated mitochondria loaded with Ca^{2+}, and then exposed to oxidants. In studies using intact cells, experimental evidence to support the release of Ca^{2+} from the mitochondrial matrix is indirect and derives from the Ca^{2+} pool releasable with the protonophore carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone FCCP (Bellomo et al., 1982; Kass et al., 1992). It might be argued that such an indirect measurement cannot accurately reflect the true changes in mitochondrial calcium ion concentration ($[Ca^{2+}]_m$) since FCCP rapidly lowers cellular ATP levels, with consequences on Ca^{2+} handling. Thus the present study utilized cells expressing the recombinant Ca^{2+}-sensitive photoprotein aequorin exclusively in the mitochondria (Rizzuto et al., 1992) in order to assess directly changes in $[Ca^{2+}]_m$ in intact endothelial cells exposed to oxidants. When oxidative stress is imposed either with a bolus addition of H_2O_2 or the enzyme system hypoxanthine-xanthine oxidase, a rise in the $[Ca^{2+}]_m$ occurs, with a higher amplitude than that observed in the cytosol. The elevation in $[Ca^{2+}]_m$ results from a transfer of Ca^{2+} secondary to an increase in cytosolic free calcium concentration ($[Ca^{2+}]_c$) via the uniporter, and an inhibition of the Ca^{2+}/Na^+ electroneutral exchanger. This finding distinguishes the present results from earlier descriptions of reversible Ca^{2+} loss from the mitochondrial matrix upon exposure to oxidants.

MATERIALS AND METHODS

Chemicals and reagents

Hypoxanthine (HX), xanthine oxidase (XO), H_2O_2, bovine liver catalase, FCCP, ruthenium red and cyclosporin A were purchased from Sigma Chemical Co. (St Louis, MO, USA). Saponin was from Merck (Dietikon, Switzerland). RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were from Gibco BRL (Basel, Switzerland). Fetal calf serum was from Seromed (Berlin, Germany). Coelenterazine, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) and the mitochondrial marker Mitotracker were obtained from Molecular Probes Europe (Leiden, The Netherlands). The CellPhect Transfection kit was from Pharmacia Biotech (Dübendorf, Switzerland). All other reagents were of analytical grade and came from Sigma or Fluka (Buchs, Switzerland). 7-chloro-3,5-dihydro-5-phenyl-1H-4.1-benzothiazepine-2-on (CGP-37157) was a kind gift from Dr A. De Pover (Novartis, Basel, Switzerland).

Cell culture

ECV304, a kind gift from Dr K. Takahashi (Takahashi et al., 1990), is an endothelial cell line originating from human umbilical vein endothelial cells by spontaneous transformation. This cell line displays endothelium-specific Weibel-Palade bodies and angiotensin-converting enzyme activity. The cells were grown in RPMI 1640 supplemented with 25 mM Hepes, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS).

Cytosolic and mitochondrially targeted aequorin

The plasmid containing the cDNA encoding apoaequorin comprises a hemagglutinin epitope and is referred to as cytosolic aequorin (cytAEQ). The mitochondrially targeted cDNA construct encodes the targeting presequence of subunit VIII of human cytochrome c oxidase, the HA1 hemagglutinin epitope and the whole photoprotein (mitAEQ). Both constructs were generous gifts from Drs Pozzan and Rizzuto and have been previously described in detail (Rizzuto et al., 1992; Brini et al., 1995).

Transfection, aequorin reconstitution and measurement

Cells were seeded onto 13 mm-diameter glass coverslips. When the cells reached ~70-80% density, transient transfection was performed with a standard calcium phosphate, according to the manufacturer's instructions. Briefly, 2.5 μg of plasmid in 12.5 μl H_2O was added under vortexing to 12.5 μl of 2× Hepes buffer (0.1 M Hepes, 0.5 M $CaCl_2$, pH 7). After a 10 minute incubation at room temperature, 25 μl of 2× HSB (0.28 M NaCl, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4, 0.05 M Hepes, pH 7) was added dropwise while vortexing, and the mixture was allowed to stand at room temperature for another 15 minutes. This was then transferred dropwise to a coverslip in 500 μl of DMEM containing 10% FCS in each well of a four-well plate. Cells were incubated with the DNA/calcium phosphate precipitate for 16-20 hours; the medium was then removed and replaced with fresh RPMI supplemented with 10% FCS. The cells were used on the next day. Transfection efficiency, estimated by scoring anti-aequorin antibody positive cells, was ~25%. Similar transfection efficiency was obtained by scoring the number of X-gal positive cells following transfection with a plasmid containing a lacZ gene driven by the RSV promoter under the same conditions. The functional photoprotein was reconstituted in situ by incubating, before the experiment, the cells with 2.5 μM of the prosthetic group coelenterazine for at least 2 hours at 37°C in RPMI. The excess coelenterazine was washed away by perfusing the cells 2-4 minutes prior to recording.

Experimental conditions and $[Ca^{2+}]$ measurements

During the experiments, cells were continously superfused with Krebs Hepes buffer (KH) containing: 120 mM NaCl, 4.75 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5 mM glucose and 25 mM Hepes, pH 7.4. Ca^{2+}-free buffer contained no $CaCl_2$ but 1 mM EGTA. Extracellular O_2^- was generated by the HX-XO system: in the presence of 4 mM HX, the concentration of XO was adjusted to an initial production of 10 nmoles O_2^-/ml/minute by following the rate of reduction of ferricytochrome C with a double beam spectrophotometer at 550 nm (Dreher et al., 1995). Exposure to HX-XO and H_2O_2 was made by perfusing in KH and by stopped flow. Light emission was measured in a purpose-built luminometer and calibrated as $[Ca^{2+}]$ (Rizzuto et al., 1992; Brini et al., 1995). At the end of each experiment, the cells were lysed by perfusing them with a hypoosmotic medium containing 10 mM $CaCl_2$, in order to expose all the cellular aequorin to a high $[Ca^{2+}]$.

Cell permeabilization

The cells were washed with Ca^{2+}-free/EGTA KH and then incubated for 10 minutes at 37°C with 100 μl of a prewarmed intracellular buffer adjusted to ~100 nM free calcium (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM Hepes, pH 7, 1 mM ATP, 10.2 mM EGTA, 1.65 mM $CaCl_2$) containing 20 μg/ml saponin (Van den Eijnden-Schrauwen et al., 1997). Permeabilization was assessed by trypan blue staining which revealed ~95% positively stained cells. For $[Ca^{2+}]$ measurement, perfusion was started with the same low Ca^{2+} intracellular buffer for 3-5 minutes, and then switched to an intracellular buffer adjusted to 500 nM free calcium (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM Hepes, pH 7, 1 mM ATP, 10.2 mM EGTA, 6.67 mM $CaCl_2$).

Immunolocalization of cytosolic and mitochondrially targeted aequorin

Transfected cells were washed 3-4 times with phosphate buffer saline

(PBS), fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature, and further washed 3-4 times with PBS. Permeabilization of cell membranes was obtained with a 10 minute incubation with 0.5% Triton X-100 in PBS, followed by a 15 minute incubation with 0.1% bovine serum albumin in PBS. The cells were then incubated for 2 hours at room temperature with a rabbit polyclonal anti-aequorin serum in PBS (1:100), washed 3-4 times with PBS, and then stained with a sheep fluorescein labelled anti-rabbit IgG antibody. Fluorescence was then observed with a microsocope and photographed with a Kodak Ektachrome 400 ASA film. The mitochondria in situ were identified with the use of the mitochondrial marker Mitotracker. To this end, the cells were incubated with 250 nM of the rhodamine-labeled Mitotracker in culture medium for 30 minutes at 37°C in the dark prior to fixation and incubation with anti-aequorin antibody.

Cytofluorimetric analysis of mitochondrial membrane potential $\Delta\Psi_m$

$\Delta\psi_m$ variations were analyzed with the cyanide JC-1 (Smiley et al., 1991; Cossarizza et al., 1993). Cells were incubated in KH containing 10 μg/ml of JC-1 in the dark at 37°C for 15 minutes. At the end of the incubation period, the cells were washed twice with PBS, trypsinized and resuspended in KH buffer (0.5×10^6 cells/ml). The cells were exposed to HX-XO (10 and 20 nmoles O_2^-/ml/minute) or H_2O_2 (1 and 5 mM) for the times indicated in Table 1, and immediately analyzed by flow cytometry (Cossarizza et al., 1993). 10^4 cells were analyzed per sample.

Measurement of ATP levels

ECV304 cells were incubated with 1 mM H_2O_2 or 10 nmoles O_2^-/ml/minute for 5, 10, 30 and 60 minutes. At the completion of the experiments, cells were scraped with a rubber policeman in 10% perchloric acid, placed on ice for 15 minutes and then centrifuged at 10000 rpm. The supernatants were collected and stored at –20°C until assayed for ATP with the luciferin-luciferase method as previously described (Junod et al., 1989). Light emission was recorded with a Packard β counter accepting signals out of coincidence. The pellets were dissolved in 0.8 N NaOH, and assayed for protein by the method of Lowry.

Statistical analysis

The significance of the difference between two groups was obtained using Student's *t*-test for unpaired data. Results are expressed as means ± s.e.m.

RESULTS

Immunolocalization of cytosolic and mitochondrially targeted aequorin

The subcellular localization of cytosolic aequorin (cytAEQ) and mitochondrially targeted aequorin (mitAEQ) was verified by immunofluorescence using a rabbit polyclonal antibody to aequorin. As shown in Fig. 1, in transfected cells with moderate expression of cytAEQ, the probe was confined mostly to the cytosol (B), while in the cell with high expression of aequorin, the probe appeared to be present also in the nucleus, due probably to the intense staining of the cytosol englobing the nucleus. In cells transfected with mitAEQ, the probe appeared exclusively localized in the mitochondria (C), since the fluorescent staining pattern almost completely coincided with that obtained with the mitochondrial marker Mitotracker (D).

Effects of addition of a bolus H_2O_2 on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in intact cells

H_2O_2 (1 mM) evoked a rise of $[Ca^{2+}]$ in the mitochondria from

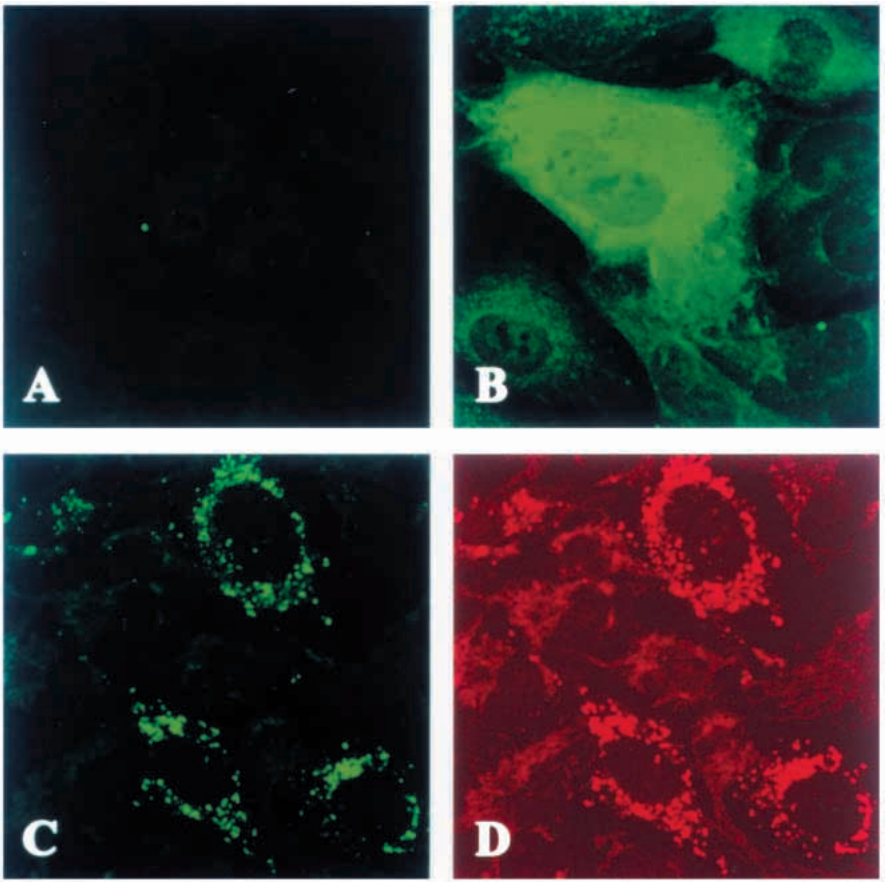

Fig. 1. Immunolocalization of cytosolic aequorin (cytoAEQ) and mitochondrially targeted aequorin (mitAEQ) in transiently transfected cells. (A and B) Cells transfected with cytoAEQ were probed with preimmune sera (A) or with a rabbit polyclonal antibody raised against the full-length protein aequorin (B), and visualized with FITC-labeled anti-rabbit antibodies. (C and D) Cells transfected with mitAEQ were probed with anti-aequorin antibody and stained with FITC-labeled anti-rabbit antibodies (C) and the mitochondrial marker Mitotracker (D).

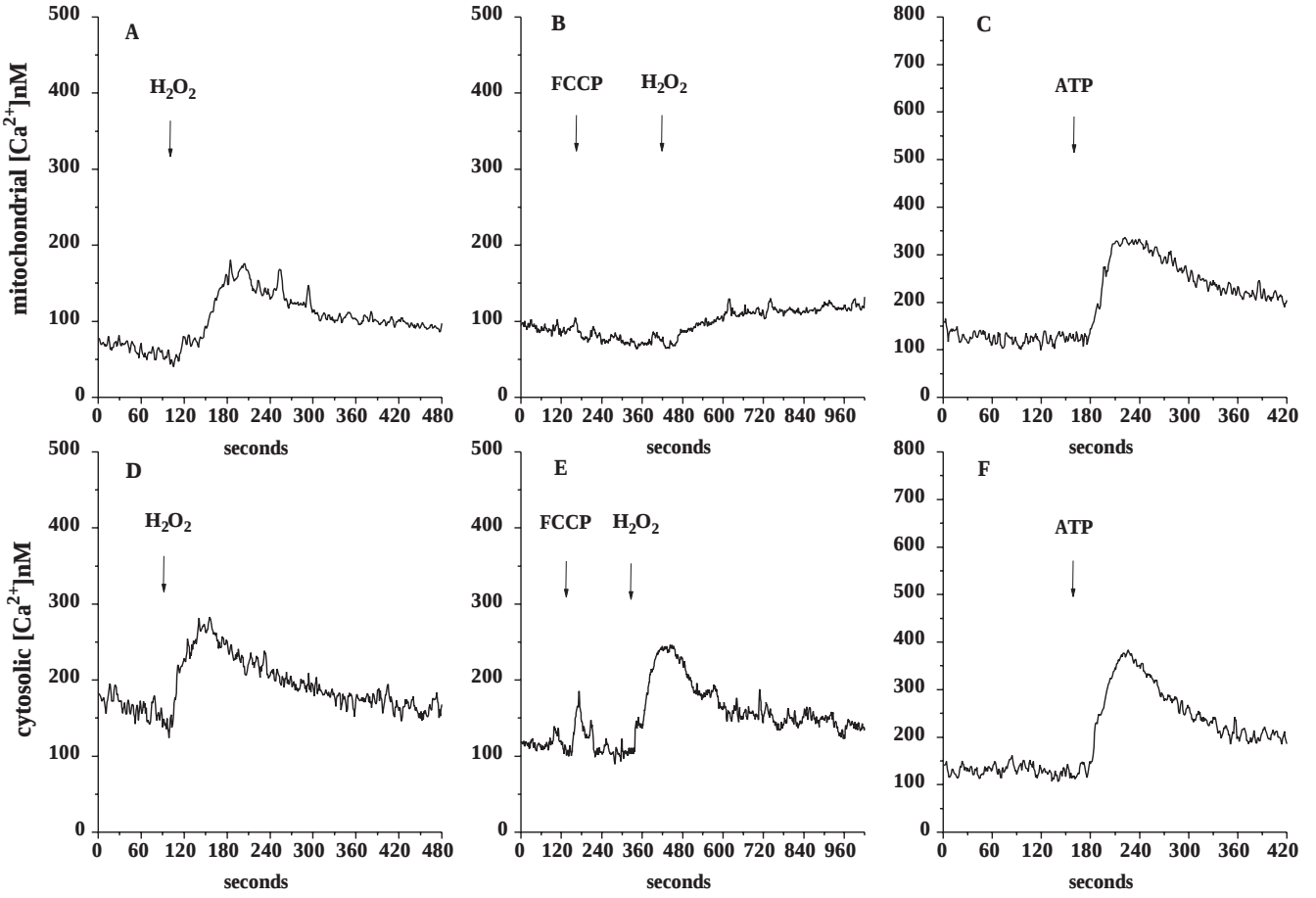

Fig. 2. $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ changes in ECV304 cells in response to 1 mM H_2O_2 in the absence (A and D) and presence (B and E) of 5 μM FCCP, and to 12.5 μM ATP (C and F). The perifusion medium contained 2.5 mM $CaCl_2$. Tracings shown are representative of 8-12 independent experiments.

85±11 nM to 214±21 nM (175±21% increase; n=12), followed by a slowly declining plateau of 128±13 nM (64±13% increase) at 5 minutes exposure (Fig. 2A). The profile of the H_2O_2-mediated Ca^{2+} signal in the mitochondria was almost identical to that observed in the cytosol: $[Ca^{2+}]_c$ increased from 142±15 nM to a peak level of 291±28 nM (110±10% increase), with a plateau level of 173±17 nM (24±6% increase) at 5 minutes of exposure (Fig. 2D). The time for $[Ca^{2+}]$ to peak after the addition of H_2O_2 was 66±4 seconds and 48±4 seconds in the mitochondria and the cytosol, respectively (n=12, P<0.05). The addition of the mitochondrial uncoupler FCCP abolished the increase in $[Ca^{2+}]_m$ (Fig. 2B) while not significantly affecting the $[Ca^{2+}]_c$ peak evoked by H_2O_2 (Fig. 2E). These results provide evidence that the Ca^{2+} signal induced by H_2O_2 occurs first in the cytosol, and is then relayed to the mitochondria. For comparison, ATP which binds the purinergic P2Y receptors and then generates inositol 1,4,5-trisphosphate (IP_3), was used (Conant et al., 1998). As shown in Fig. 2C-F, ATP (12.5 μM) caused similar biphasic increases in $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$.

The dose-response curves in Fig. 3 show that the amplitude of the Ca^{2+} response increases with increasing H_2O_2

Table 1. Effects of H_2O_2 and HX-XO on mitochondrial membrane potential

	Time (minutes)					
	1	5	10	20	30	60
Control	76±4	–	73±6	–	72±3	72±3
H_2O_2 1 mM	79±2	79±2	76±1	75±3	75±2	70±0.8
H_2O_2 5 mM	79±2	79±2	75±1	74±2	72±2	70±2
XO10	75±4	75±4	73±4	73±5	75±4	70±6
XO20	76±4	75±4	76±4	75±4	74±4	70±5
FCCP 5 μM	31±6	17±4	11±3	5±1	4±1	1±0.3

Cytofluorimetric analysis of cells stained with JC-1 and exposed to H_2O_2 (1 and 5 mM), HX-XO that generates 10 nmoles O_2^-/ml/minute (XO10) and 20 nmoles O_2^-/ml/minute (XO20), and FCCP (5 μM) for the times indicated. The values (means ± s.e.m. of 4 separate experiments) represent the percentage of cells exhibiting orange/red fluorescence, e.g. polarized mitochondria.

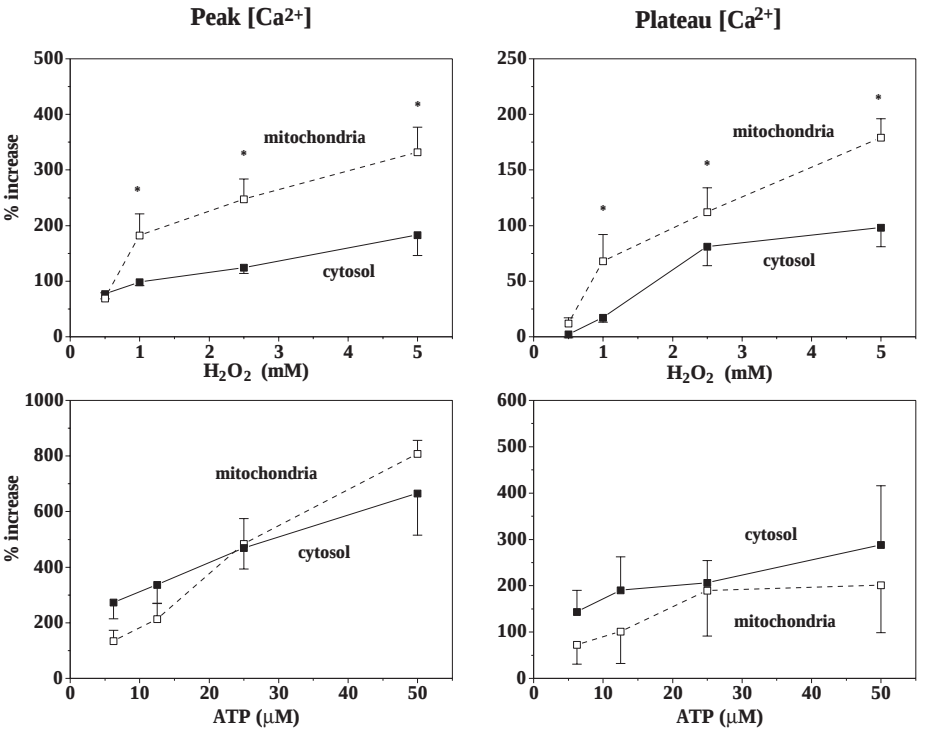

Fig. 3. Effects of increasing concentrations of H_2O_2 and ATP on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$. Results are expressed as the percentage of increase over basal levels. *P<0.05 compared with $[Ca^{2+}]_c$ values, n=6-12. Left panels show the peak Ca^{2+} levels, and right panels show the Ca^{2+} values of the plateau phase 5 minutes after stimulation.

concentrations, and that H_2O_2 elicits a larger peak $[Ca^{2+}]$ and a larger plateau $[Ca^{2+}]$ (upper panels) in the mitochondria than in the cytosol (n=6-12, P<0.05). Application of the IP_3-generating agonist ATP (6.25-50 μM) to cells superfused with Ca^{2+}-containing medium also increased mitochondrial and cytosolic $[Ca^{2+}]$ in a dose-dependent manner with a nearly maximum effect at 25 μM (Fig. 3, lower panels). However, in contrast to H_2O_2, the ATP-evoked rise in $[Ca^{2+}]_m$ was comparable to the $[Ca^{2+}]_c$ response. Thus, the larger increase of $[Ca^{2+}]_m$ evoked by H_2O_2 can be attributed to either the close juxtaposition of the mitochondria with a Ca^{2+} pool sensitive to H_2O_2, but not to IP_3 generated by ATP. Alternatively, an inhibitory effect of H_2O_2 on the mitochondrial Ca^{2+} extrusion system may be involved.

To address the first possibility, cells were stimulated in Ca^{2+}-free medium containing 1 mM EGTA. As shown in Fig. 4, left panels, under this condition of suppressed Ca^{2+} influx from external medium, the plateau phase of elevated $[Ca^{2+}]$ was abolished in the cytosol (8±4% increase, n=8, P<0.05 when compared to that observed in the presence of external calcium). In contrast, in the mitochondria, the plateau phase remained high at 5 minutes post-stimulation (79±12% increase, n=8) and was not significantly different from that observed in cells bathed in Ca^{2+}-containing medium (64±13% increase). Likewise, no Ca^{2+} response in the cytosol was observed when H_2O_2 was added after a challenge with histamine (100 μM) and ATP (100 μM) which mobilize Ca^{2+} from endoplasmic reticulum (10±5% increase, n=7), whereas a significant rise was still observed in the mitochondria (63±12 % increase, n=7, P<0.05, Fig. 4, middle panels). Similarly, when cells were first stimulated by the Ca^{2+}-ATPase inhibitor CPA (40 μM), subsequent addition of H_2O_2 failed to elicit any significant Ca^{2+} response in the cytosol (13±4% increase; n=5) but still caused a rise in $[Ca^{2+}]_m$ (93±16% increase, n=5, P<0.05, Fig. 4, right panels). The observation that the Ca^{2+} response was maintained in the mitochondria while being totally abolished in the cytosol argues for an accumulation of Ca^{2+} within the mitochondria as a result of a decreased rate of efflux of Ca^{2+} from the organelle via the exchangers.

To test more directly this hypothesis, cells were continously perifused with 10 μM CGP37157, a specific and potent inhibitor of the mitochondrial Ca^{2+}/Na^+ exchanger (Cox and Matlib, 1993a,b). The rationale of this approach is that CGP37157 treatment should result in a larger increase in $[Ca^{2+}]_m$ after stimulation with a Ca^{2+} mobilizing agonist or the Ca^{2+}-ATPase inhibitor CPA, but not after stimulation with H_2O_2 since the latter is also proposed to act similarly by inhibiting the mitochondrial Ca^{2+} extrusion system. This is indeed the case. Fig. 5A shows that upon stimulation with 50 μM histamine (a concentration chosen to observe the potentiating effect of the inhibitor), CGP37157 caused a marked increase in the peak $[Ca^{2+}]_m$ (775±67% increase in the presence of the inhibitor compared with 320±60% increase in the absence of the inhibitor; n=5; P<0.05). Although the initial Ca^{2+} efflux rate appeared not to be altered by CGP37157, the protracted efflux was markedly reduced (65±8% versus 8±9% increase in $[Ca^{2+}]_m$ after 5 minutes of stimulation, in the presence and absence of the inhibitor, respectively; P<0.05). Similarly, Fig. 5B shows that the peak $[Ca^{2+}]_m$ increase induced by 2.5 μM CPA was substantially higher in the presence of CGP37157: 602±112% and 276±42% increase in the presence and absence of the inhibitor, respectively (n=7, P<0.05). The subsequent plateau phase also remained higher in the presence of CGP37157: 360±46% and 109±14% increase in the presence and absence of the inhibitor (n=7, P<0.05). In contrast with results obtained with histamine and CPA, no difference could be observed in the amplitude of the $[Ca^{2+}]_m$ rises after addition of 2.5 mM H_2O_2 in the presence and absence of CGP37157 (Fig. 5C): the peak $[Ca^{2+}]_m$

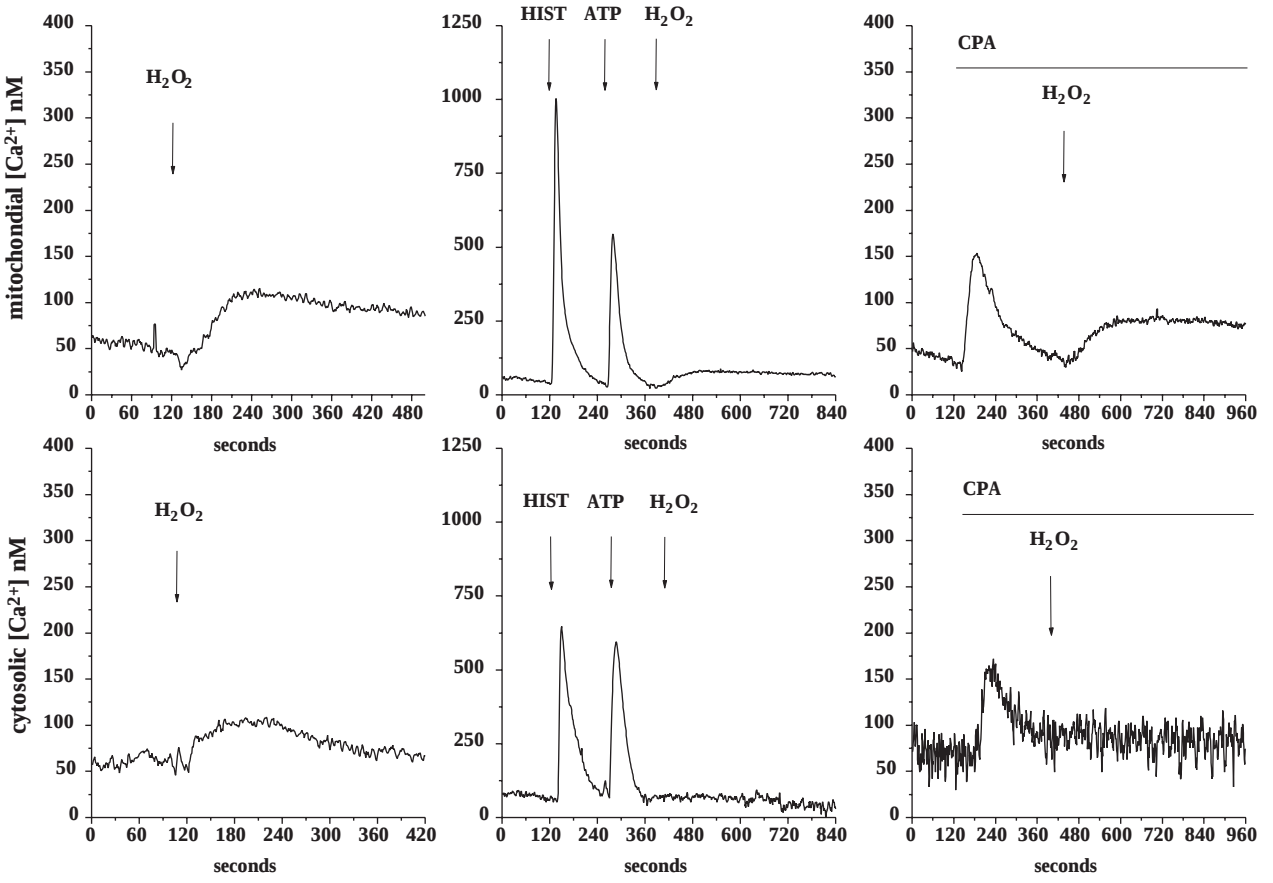

Fig. 4. Left panels show the effects of 1 mM H_2O_2 on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in Ca^{2+} free/EGTA medium. Middle and right panels depict the effects of 1 mM H_2O_2, when added after histamine (100 μM) and ATP (100 μM), or after CPA (40 μM) on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in Ca^{2+} free/EGTA medium. Tracings shown are representative of 5-7 independent experiments.

increased by 132±14% and 151±19% (n=5) in the presence and absence of the inhibitor. The plateau $[Ca^{2+}]_m$ increase at 5 minutes post-stimulation showed an 88±14% and 51±22% increase (n=5), with and without CGP37157, respectively. These results are consistent with the idea that H_2O_2 inhibits the efflux of Ca^{2+} from the mitochondria by acting on the same site as CGP37157. To test whether the lack of effect of CGP37157 was related to a loss of its activity due to oxidative attack by H_2O_2, we treated in vitro CGP37157 (10 μM) with 2.5 mM H_2O_2 for 5 minutes at room temperature, then added catalase (200 U/ml) to eliminate H_2O_2, prior to addition to the perifusion system. As shown in Fig. 5A, CGP37157 pretreated with H_2O_2 (trace c, dashed line) was as potent as the untreated product in inhibiting the Ca^{2+}/Na^+ exchanger (trace b, dotted line).

Effects of H_2O_2 and CGP37157 on $[Ca^{2+}]_m$ in permeabilized cells

To confirm that H_2O_2 was inhibiting the process of Na^+/Ca^{2+} exchange, we studied this effect directly by the use of permeabilized cells perfused with an intracellular-like buffer adjusted to 100 or 500 nM free calcium corresponding to basal and stimulatory levels in intact cells.This was done in the absence or presence of H_2O_2 or CGP37157. This approach allows the clamping of $[Ca^{2+}]_c$ in order to study the mitochondrial Ca^{2+} handling in an environment strictly controlled in terms of ionic species, pH and ATP concentration. H_2O_2 (1 mM) did not change the free Ca^{2+} concentration or pH of the buffer. In these experiments, lower concentrations of H_2O_2 and CGP37157 were used since mitochondria are expected to be more accessible in permeabilized cells compared to intact cells. As shown in Fig. 6A, in permeabilized cells, switching from 100 nM to 500 nM Ca^{2+} increased $[Ca^{2+}]_m$ from 102±4 nM to 434±21 nM after 3 minutes (n=15). This increase was inhibited by 90% by ruthenium red, a blocker of Ca^{2+} uniporter. Fig. 6B shows that in cells perfused with an intracellular-like buffer containing 100 nM free calcium, 1 mM H_2O_2 promoted a slight but consistent increase in $[Ca^{2+}]_m$ (from 99±1.5 to 180±14 nM; n=6; P<0.05) similar to that induced by 5 μM CGP37157 (from 109±6 to 214±14 nM; n=4; P<0.05). The increase in $[Ca^{2+}]_m$ seen at 500 nM Ca^{2+} was not further enhanced by the presence of H_2O_2 or CGP37157: 421±21 and 478±50 nM, respectively (see Fig. 6, B compared to A). Similar mitochondrial Ca^{2+} responses were observed when cells were perfused at 500 nM calcium, then returned to 100 nM Ca^{2+} in the continued presence of H_2O_2 or CGP37157 (Fig. 6C). The rate of Ca^{2+} release promoted by the decrease of the extramitochondrial Ca^{2+} was slower in the presence of H_2O_2 or CGP37157: after 4 minutes, the new baseline levels were 196±26 nM (P<0.05 compared with

control cells; n=4), 217±37 nM (P<0.05 compared with control cells; n=4) and 112±5 (n=15), respectively, for H_2O_2, CGP37157 and control cells. The effect of H_2O_2 depends on Ca^{2+} cycling across the mitochondrial membrane since the H_2O_2-induced increase in $[Ca^{2+}]_m$ was completely inhibited by ruthenium red (Fig. 6B, dashed line). These results strengthen the hypothesis that H_2O_2 inhibits the Ca^{2+}/Na^+ electroneutral exchanger.

Effects of HX-XO on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in intact cells

As shown in Fig. 7, left panels, addition of a dose of XO which produces 10 nmoles O_2^-/ml/minute resulted in a rapid increase in $[Ca^{2+}]_m$, from a basal value of 74±6 nM to a peak level of 248±20 nM (254±27% increase, n=20), with a subsequent sustained plateau of 178±15 nM (120±15% increase) at 5 minutes. The $[Ca^{2+}]_c$ increased from a resting level of 102±9 nM to a peak value of 280±24 nM (184±23% increase, n=8), followed by a plateau level of 175±19 nM (71±14% increase). There was a latency of 59±2 seconds and 53±2 seconds before the peak $[Ca^{2+}]$ signal in the mitochondria and the cytosol, respectively (P>0.05). Pretreatment with 5 μM of the mitochondrial uncoupler FCCP completely prevented the increase in $[Ca^{2+}]_m$, but had no significant effect on the increase in $[Ca^{2+}]_c$ (data not shown).

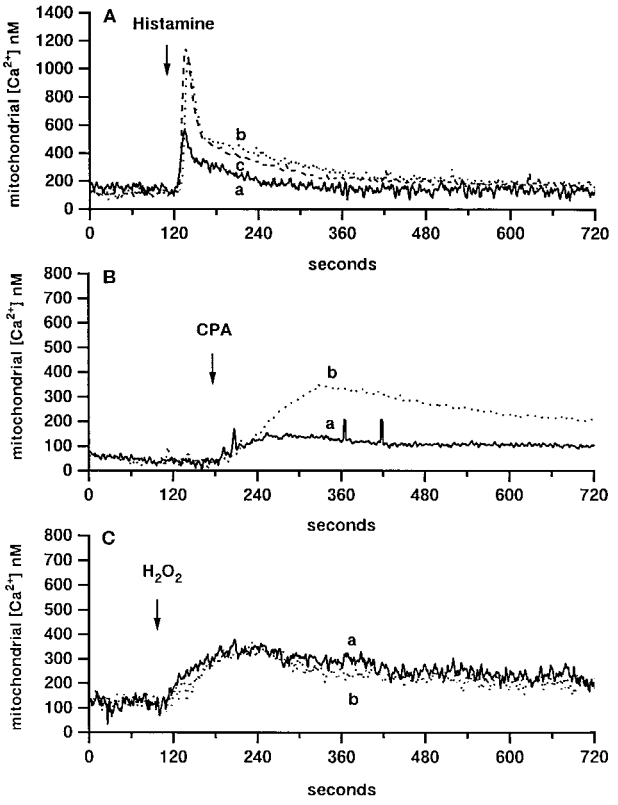

Fig. 5. Effects of CGP37157 on $[Ca^{2+}]_m$ changes induced by 50 μM histamine (A), 2.5 μM CPA (B) and 2.5 mM H_2O_2 (C) in intact cells in Ca^{2+} containing medium. Traces (a): without CGP37157; traces (b): with 10 μM CGP37157. A trace (c): CGP37157 was exposed in vitro to 2.5 mM H_2O_2 for 5 minutes at room temperature, then 200 U/ml catalase was added to eliminate H_2O_2, prior to addition to the perifusion buffer.

Fig. 7 also shows the effects of varying the concentration of XO that produces different amounts of O_2^-/ml/minute. The magnitude of response increased with increasing O_2^- concentrations, and the peak rise of $[Ca^{2+}]$ was consistently, although not significantly, higher in the mitochondrial matrix than in the cytosol (middle panel). High doses of XO (10 and 20 nmoles O_2^-/ml/minute) elicited a plateau phase of elevated $[Ca^{2+}]$ which was clearly more pronounced in the mitochondria (n=6, P<0.05, right panel).

These data indicate that the Ca^{2+} signal induced by HX-XO occurred first in the cytosol, and was then relayed to the mitochondria, supporting the hypothesis that O_2^-/H_2O_2 may also inhibit the mitochondrial Ca^{2+} efflux process. We directly addressed the latter issue in the experiment presented in Fig. 8, which shows the effects of the antiporter inhibitor CGP37157. Inhibition of the antiporter by CGP37157 had no additive effect on the $[Ca^{2+}]_m$ increase in response to HX-XO, suggesting a common site of action. As for H_2O_2, pretreatment

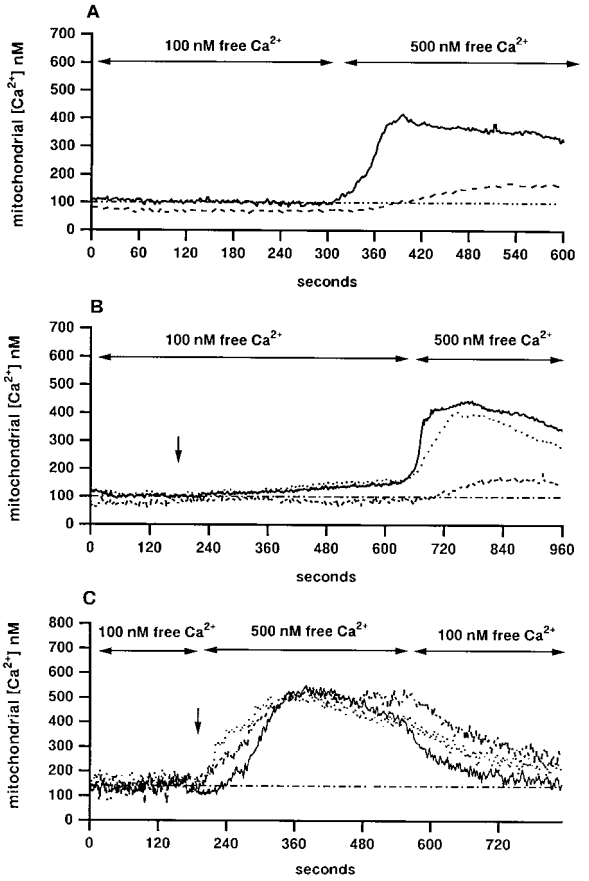

Fig. 6. Effects of H_2O_2 and CGP37157 on $[Ca^{2+}]_m$ in permeabilized cells. (A) Cells were perfused first with an intracellular-like buffer containing 100 nM free Ca^{2+} then with a 500 nM free Ca^{2+} buffer in the absence (solid line) or presence of 50 μM ruthenium red (dashed line). (B) Conditions as in A with the addition of 1 mM H_2O_2 (dotted line) or 5 μM CGP37157 (solid line) indicated by the arrow. Dashed line: 1 mM H_2O_2 in the presence of 50 μM ruthenium red. (C) permeabilized cells were initially perfused with 100 nM free Ca^{2+} buffer. Where indicated, the medium was changed to a 500 nM free Ca^{2+} buffer, then again to a 100 nM free Ca^{2+} buffer, containing 1 mM H_2O_2 (dotted line), 5 μM CGP37157 (dashed line) or nothing (solid line). Horizontal lines represent baseline levels.

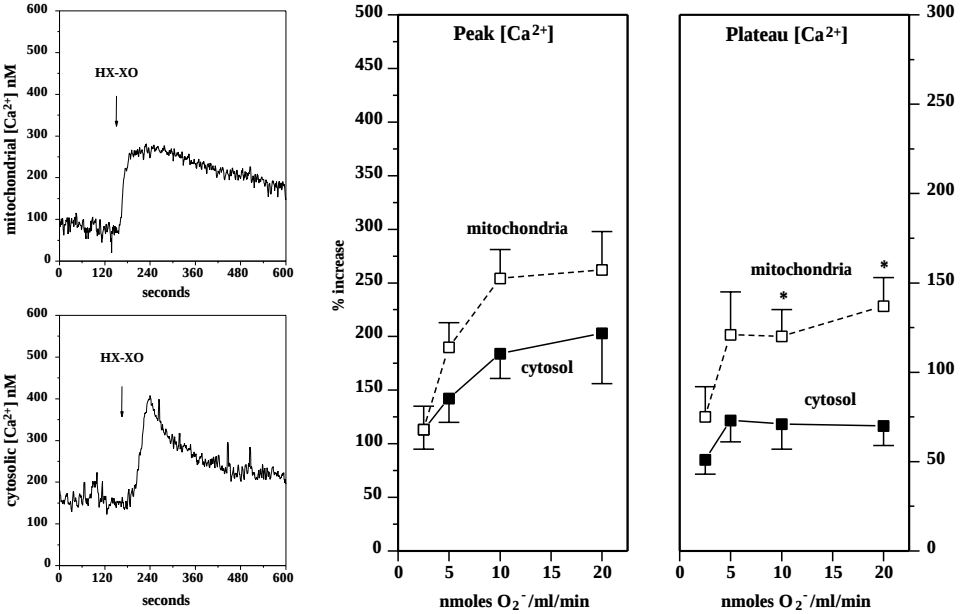

Fig. 7. Left panels: representative tracings showing the effects of a flux of 10 nmoles O_2^-/ml/minute on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in Ca^{2+}-containing medium. Middle and right panels show the effects of various concentrations of XO that generate different amounts of O_2^- on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$.Values are expressed as the percentage of increase over basal levels. **P*<0.05 compared with $[Ca^{2+}]_c$ values, *n*=6-8. The middle panel depicts the peak Ca^{2+} levels, and the right panel shows the Ca^{2+} values of the plateau phase 5 minutes after stimulation.

of CGP37157 with HX-XO did not impair the activity of the product on $[Ca^{2+}]_m$ increased by the agonist (data not shown).

Effects of H_2O_2 and HX-XO on mitochondrial transmembrane potential $\Delta\psi_m$

The staining of cells with the lipophilic cation JC-1 permits measurement of $\Delta\psi_m$ within intact cells. JC-1 has the unique property of forming J-aggregates locally and spontaneously under high mitochondrial $\Delta\psi_m$, which are red fluorescent, whereas the monomeric form is green fluorescent. The data in Table 1 show that about 80% of normal cells exhibited orange/red fluorescence, and the percentage of cells with polarized mitochondria was similar in control cells and in cells exposed to H_2O_2 or HX-XO for up to 60 minutes. FCCP, a protonophore that abolishes the electrochemical gradient, dissipated $\Delta\psi_m$ completely, as shown by a rapid disappearance of the J-aggregate fluorescence.

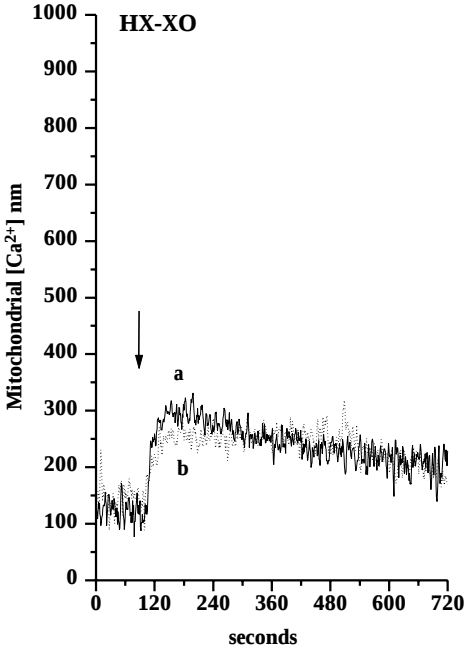

Fig. 8. Effect of CGP37157 on $[Ca^{2+}]_m$ changes induced by HX-XO (10 nmoles O_2^-/ml/minute) in Ca^{2+}-containing medium. Trace (a): without CGP37157; trace (b): with 10 μM CGP37157.

Effects of H_2O_2 and HX-XO on intracellular ATP content

A 10 minute exposure to 1 mM H_2O_2 did not affect endothelial cell ATP content. After 30 minutes exposure to 1 mM H_2O_2, ATP levels declined from 35.5±1.5 pmol/μg protein to 14.5±4 pmol/μg protein (*n*=3; *P*<0.05). After 1 hour exposure, intracellular ATP levels declined further to 9±2 pmol/μg protein (*n*=4; *P*<0.05). When endothelial cells were exposed to HX-XO (10 nmoles O_2^-/ml/minute), ATP levels remained unchanged for up to 1 hour of exposure.

DISCUSSION

In the present report, we show that, in the endothelial cell line ECV304, a bolus addition of H_2O_2, and the superoxide anions generated by the enzyme sytem HX-XO induced a rise of $[Ca^{2+}]$ in the mitochondria, the amplitude of which always exceeded that of the $[Ca^{2+}]_c$ increase. This increase in $[Ca^{2+}]_m$ results from a transfer of Ca^{2+} secondary to increased $[Ca^{2+}]_c$ via the electrogenic uniporter, and a decrease in the rate of Ca^{2+} efflux from the mitochondria via the Ca^{2+}/Na^+ electroneutral exchanger. To our knowledge, this is the first report on the Ca^{2+} handling in the mitochondria within intact cells exposed to an oxidant insult.

The primary effect of H_2O_2 and HX-XO is an increase of $[Ca^{2+}]$ in the cytosol secondary to Ca^{2+} release from stores with consequent stimulation of capacitative Ca^{2+} influx (Doan et al., 1994). This increase in $[Ca^{2+}]_c$ is then rapidly relayed to the mitochondria via the uniporter process. This notion is supported by the following observations: (1) the peak rise of $[Ca^{2+}]_m$ lagged

behind that of $[Ca^{2+}]_c$; (2) in the presence of the protonophore FCCP that dissipates $\Delta\psi_m$, H_2O_2 and HX-XO failed to elicit any detectable increase of $[Ca^{2+}]_m$; (3) the mitochondria remained polarized during the 60 minutes of exposure to oxidants; and (4) in permeabilized cells perfused with an intracellular-like buffer containing 500 nM free Ca^{2+}, H_2O_2-induced Ca^{2+} uptake in mitochondria was largely prevented by ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uniporter.

The other effect of H_2O_2 and HX-XO is an inhibition of the Ca^{2+}/Na^+ exchange process. There are several lines of evidence, albeit indirect, that support this concept. First, the peak $[Ca^{2+}]$ increase and the plateau phase of elevated $[Ca^{2+}]$ evoked by H_2O_2 and HX-XO were consistently higher in the mitochondria than in the cytosol, especially at high doses of the oxidants. Second, in cells bathed in Ca^{2+}-free medium, the plateau $[Ca^{2+}]$ evoked by H_2O_2 was abolished in the cytosol but not in the mitochondria. Third, a challenge with H_2O_2 after CPA and agonists failed to elicit any detectable response in the cytosol, while still producing a rise of Ca^{2+} in the mitochondria. The existence of microdomains of high $[Ca^{2+}]$ in the regions surrounding the IP_3 sensitive calcium release sites of the endoplasmic reticulum (Rizzuto et al., 1993, 1994) could be another potential mechanism to explain a proportionally greater increase in the mitochondrial $[Ca^{2+}]$ over the cytosolic $[Ca^{2+}]$. The results obtained with the IP_3-generating agonist ATP and the Ca^{2+}-ATPase inhibitor CPA argue against this possibility. In fact, in contrast to the Ca^{2+} response to H_2O_2 and HX-XO, the increase of $[Ca^{2+}]_m$ in response to ATP and CPA was comparable to that of $[Ca^{2+}]_c$.This is in accordance with the observation that, in ECV304 cells, the mitochondria are not, in contrast to other cell lines, located in sufficiently close proximity to the endoplasmic reticulum to detect any $[Ca^{2+}]_c$ microdomains which would occur at the time of agonist-stimulated Ca^{2+} release (Lawrie at al., 1996). The fourth line of evidence that oxidants inhibit the mitochondrial Ca^{2+} extrusion system comes from experiments using the inhibitor of the mitochondrial Ca^{2+}/Na^+ exchanger, CGP37157. A decrease in the activity of the Ca^{2+}/Na^+ exchanger is expected to lead to an increase in the basal level of matrix free Ca^{2+} and possibly to a greater increase in the matrix free Ca^{2+} upon increasing the stimulation rate. In intact cells, CGP37157 did not potentiate the effects of H_2O_2 and HX-XO on $[Ca^{2+}]_m$, while exhibiting a considerably potentiating effect on the increase of $[Ca^{2+}]_m$ in response to histamine and CPA. The absence of additive effects of H_2O_2 and CGP37157 on $[Ca^{2+}]_m$ argues for a common site of action, e.g. the Ca^{2+}/Na^+ exchanger. This is further corroborated by the results obtained in permeabilized cells. Even in conditions of clamped cytosolic $[Ca^{2+}]$ at the resting level of 100 nM, H_2O_2 increased $[Ca^{2+}]_m$. This effect was mimicked by CGP37157. Moreover, when the efflux of Ca^{2+} from the mitochondrial matrix was promoted by changing $[Ca^{2+}]_c$ from 500 nM to 100 nM, H_2O_2, like CGP37157, slowered the efflux. These results demonstrate the direct effect of H_2O_2 on $[Ca^{2+}]_m$ handling independently of $[Ca^{2+}]_c$ changes, and strongly suggest Ca^{2+}/Na^+ antiporter as a target for H_2O_2. It may appear surprizing that the increase in $[Ca^{2+}]_m$ seen at 500 nM Ca^{2+} was not further enhanced by H_2O_2 or CGP37157. It could be speculated that the diminished Ca^{2+} influx due to desensitization of the uniporter results in decreased activity of Ca^{2+}/Na^+ exchanger thereby explaining the lack of effect of H_2O_2 and CGP37157. Such desensitization has been observed under similar conditions in permeabilized INS-1 insulinoma cells (Maechler et al., 1998). Alternatively, in permeabilized cells bathed in 500 nM free Ca^{2+} buffer, activation of the $2H^+/Ca^{2+}$ antiporter, the other electroneutral exchanger, cannot be excluded. Regardless of the mechanism, the fact that the same effect was observed when H_2O_2 was used in place of CGP37157 strongly suggests the Ca^{2+}/Na^+ antiporter as the site of action of H_2O_2. Whether the oxidant-induced inactivation of the Ca^{2+}/Na^+ exchanger was due to direct oxidative damage to the antiporter requires further investigation.

Our findings are, at first sight, in contradiction to earlier work reporting a loss of Ca^{2+} from the mitochondrial matrix upon exposure to oxidants. In these previous studies, oxidizing agents induced Ca^{2+} release from isolated mitochondria secondary to the opening of the transition pore (Crompton et al., 1988; Fagian et al., 1990) or via a selective oxidant-dependent pathway (Schlegel et al., 1992), with a resultant collapse of membrane potential. In the present work, H_2O_2 and HX-XO did not damage the mitochondria, as demonstrated by preserved $\Delta\psi_m$, a reliable criterion for functional integrity of mitochondria, as well as normal ATP levels, at least during the first 30 minutes of exposure. The decrease in ATP levels observed after 30 minutes exposure to H_2O_2 might result from a decrease in NAD^+ content due to the stimulation of poly (ADP)ribose polymerase activity following DNA damage by the oxidant (Schraufstätter et al., 1986; Hyslop et al., 1988). It should be pointed out that most of the previous studies were carried out using isolated mitochondria with supraphysiological Ca^{2+} loads, while the present study monitored Ca^{2+} changes in mitochondria in situ in intact or permeabilized cells exposed to oxidants. It is also important to bear in mind that the results obtained with isolated mitochondria excluded the participation of specific antioxidant enzymes and other thiol compounds such as glutathione and thioredoxin present in the cytosolic compartment which may well scavenge the oxygen metabolites before they can damage the mitochondria in intact cells. This may explain, at least in part, the deleterious effects of oxidants observed in vitro on isolated mitochondria loaded with Ca^{2+}. However, in early work with intact hepatocytes, Orrenius and coworkers reported that tert-butyl-hydroperoxide caused Ca^{2+} loss from mitochondrial and extramitochondrial compartments (Bellomo et al., 1982). Later, it was observed that, in intact hepatocytes exposed to either benzoquinone imine or cumene hydroperoxide, the mitochondrial Ca^{2+} content transiently increased during the first 15 minutes of exposure, after which a complete loss of Ca^{2+} from this organelle occurred (Kass et al., 1992). In those studies, the size of the mitochondrial Ca^{2+} pool was indirectly estimated from the FCCP-releasable Ca^{2+} pool. The pools discharged by FCCP are likely to be multiple, since H^+ ionophores that collapse internal pH gradients have been shown to release Ca^{2+} from acidic organelles (Fasolato et al., 1991; Shoshan et al., 1981; Shorte et al., 1991).

In summary, the present study provides evidence that: (i) a short term exposure to H_2O_2 or the anion superoxide generated by the enzyme system HX-XO leads to a sustained elevation of $[Ca^{2+}]_m$ secondary to $[Ca^{2+}]_c$ changes; (ii) this rise in $[Ca^{2+}]_m$ depends on Ca^{2+} uptake via the uniporter, and results from the inhibition of the mitochondrial Ca^{2+}/Na^+ antiporter; and (iii) the increase in cytosolic calcium triggered by oxidants cannot be attibuted to a release of calcium from the mitochondrial matrix, as has been suggested in the past (Bellomo et al., 1982). We propose that, in mitochondria studied in living cells: (i) Ca^{2+} accumulation by mitochondria is the primary event that occurs

under oxidative stress; and (ii) the oxidant-induced loss of mitochondrial Ca^{2+} reported by other authors is likely to be secondary to it, since opening of the transition pore is greatly favored by increased matrix Ca^{2+}. A pathological model that may be relevant to the present observation is reperfusion or reoxygenation after prolonged anoxia. This clinical condition is accompanied by generation of reactive oxygen species (Kloner et al., 1989), and is associated with massive accumulation of Ca^{2+} by mitochondria (Allen et al., 1993) coupled with permeability transition induction (Karmazyn, 1991).

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