Hydrogen peroxide attenuates store-operated calcium entry and enhances calcium extrusion in thyroid FRTL-5 cells

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Redox modulation participates in the regulation of intracellular free calcium concentration ($[Ca^{2+}]_i$) in several cell types. In thyroid cells, including FRTL-5 cells, changes in $[Ca^{2+}]_i$ regulate several important functions, including the production of H_2O_2 (hydrogen peroxide). As H_2O_2 is of crucial importance for the production of thyroid hormones, we investigated the effects of H_2O_2 on $[Ca^{2+}]_i$ in thyroid FRTL-5 cells. H_2O_2 itself did not modulate basal $[Ca^{2+}]_i$. However, H_2O_2 attenuated store-operated calcium entry evoked by thapsigargin, both in a sodium-containing buffer and in a sodium-free buffer. The effect of H_2O_2 was abrogated by the reducing agent β-mercaptoethanol. H_2O_2 also attenuated the thapsigargin-evoked entry of barium and manganese. The effect of H_2O_2 was, at least in part, mediated by activation of protein kinase C (PKC), as H_2O_2 enhanced the binding of [^{3}H]phorbol 12,13-dibutyrate. H_2O_2 also stimulated the translocation of the isoenzyme PKCϵ from the cytosolic fraction to the particulate fraction. Furthermore, H_2O_2 did not attenuate store-operated calcium entry in cells treated with staurosporine or calphostin C, or in cells with down-regulated PKC. H_2O_2 depolarized the membrane potential in bisoxonol-loaded cells and when patch-clamp in the whole-cell mode was used. The depolarization was attenuated in cells with down-regulated PKC. This depolarization, at least in part, explained the H_2O_2-evoked inhibition of calcium entry. In addition, H_2O_2 enhanced the extrusion of calcium from cells stimulated with thapsigargin and this effect was abolished in cells with down-regulated PKC and after treatment of the cells with the reducing agent β-mercaptoethanol. In conclusion H_2O_2 attenuates an increase in $[Ca^{2+}]_i$. As H_2O_2 is produced in thyroid cells in a calcium-dependent manner, our results suggest that H_2O_2 may participate in the regulation of $[Ca^{2+}]_i$ in these cells via a negative-feedback mechanism involving activation of PKC.

Key words: calcium channels, redox regulation, Ca^{2+}-ATPase, ion fluxes.

INTRODUCTION

In thyroid cells, as in several other cell types, changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) [1] regulate a multitude of cellular functions. Several reports have shown that changes in $[Ca^{2+}]_i$ evoked by ATP, carbachol, noradrenaline, and also by thyrotropin (TSH), may regulate the efflux of iodide [1–3], cellular proliferation [4,5], the expression of receptors for TSH [6] and the generation of H_2O_2 (hydrogen peroxide) [7–9].

In the thyroid, the production of thyroid hormones takes place at the external surface of the apical plasma membrane. In the iodination and coupling reaction, in which thyroid hormones are formed, H_2O_2 acts as an electron acceptor. The system generating H_2O_2 uses NADPH as a coenzyme, and together with a thyroid peroxidase regulates thyroid hormone production. Both of these enzymes are also found at the apical plasma membrane. Thus as calcium participates in the regulation of H_2O_2 production, it is an important factor in the regulation of thyroid hormone synthesis. Furthermore, it is feasible to assume that cytosolic calcium may reach high micromolar levels close to the plasma membrane as a result of both agonist-evoked release from intracellular stores and store-operated entry. At these sites the production of H_2O_2 is also substantial [10]. Although the production of H_2O_2 is an important event in the regulation of thyroid cell function, the effect of H_2O_2 itself is not well documented. In thyroid FRTL-5 cells, H_2O_2 either enhances or inhibits iodide entry, depending on the hormonal status of the cells [11,12], whereas in porcine thyroid cells H_2O_2 inhibits iodide uptake and iodine organification [13] and may induce apoptosis [14]. In other cell types, H_2O_2 appears to have a multitude of effects. In ventricular myocytes, H_2O_2 slows the inactivation of a tetrodotoxin-sensitive sodium current [15], evokes changes in $[Ca^{2+}]_i$ [16,17], and may have arrhythmic activity [18]. Furthermore, H_2O_2 has been shown to activate activator protein-1 (AP-1) and nuclear factor κB transcription factors [19], and it may evoke apoptosis in some cells [20,21].

H_2O_2 is a powerful oxidizing compound, which enhances the entry of extracellular calcium into some cell types [22,23]. Oxidizing compounds may modulate the release of sequestered calcium, by sensitizing the inositol 1,4,5-trisphosphate (IP_3) receptor at resting levels of IP_3 [24,25], and by sensitizing the ryanodine receptor [26]. Some oxidizing compounds, e.g. thimerosal, may activate calcium entry via calcium-release-activated calcium channels [27]. We have shown that thimerosal activates a Ca^{2+}-ATPase and calcium extrusion in FRTL-5 cells [28]. Oxidizing compounds may also inhibit the endoplasmic Ca^{2+}-ATPase [29]. In addition, H_2O_2 may activate protein kinase C (PKC) [30,31]; and PKC modulates several functions in FRTL-5 cells [32], including the regulation of calcium entry [33].

Reactive oxygen species, including H_2O_2, are probably formed in all cell types, but because H_2O_2 is of crucial physiological

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; $[Ca^{2+}]_o$, extracellular free calcium concentration; fura 2/AM, fura 2 acetoxymethyl ester; HBSS, Hepes-buffered saline solution; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; TSH, thyrotropin.

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importance for the function of thyroid cells, we wanted to investigate the effect of H_2O_2 on calcium fluxes in thyroid FRTL-5 cells. To obtain entry of calcium without any other receptor-evoked signalling cascades, we induced store-operated calcium entry using the Ca^{2+}-ATPase inhibitor thapsigargin. Our results show that H_2O_2 potently attenuates an increase in $[Ca^{2+}]i$ by two mechanisms: (1) a PKC-dependent depolarization of the membrane potential and a concomitant decrease of the electrochemical gradient for calcium; and (2) a PKC-dependent extrusion of calcium from the cell.

MATERIALS AND METHODS

Materials

Culture medium, serum and hormones needed for cell culture were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.), Biological Industries (Kibutz, Beit, Haemek, Israel) and Sigma (St. Louis, MO, U.S.A.). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, U.S.A.). Staurosporine and PMA were purchased from Sigma, H_2O_2 was obtained from Riedel-de Haen (Seelze, Germany) and β-mercaptoethanol was from Fluka Chemie (Buchs, Germany). Fura 2 acetoxymethyl ester (fura 2/AM), fluo 3 and bisoxonol were purchased from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin was from LC Services Corp. (Woburn, MA, U.S.A.). [^{3}H]Phorbol 12,13-dibutyrate and [γ-^{32}P]ATP were obtained from Amersham (Amersham, Bucks., UK). Phosphocellulose paper P81 was from Whatman (Maidstone, Kent, U.K.). Microtitration plates used in the phorbol dibutyrate binding assay, and the liquid scintillation cocktails Optiphase SuperMix and HiSafe 3, were purchased from Wallac (Turku, Finland). All other chemicals used were of reagent grade. Bovine TSH was a generous gift from the National Hormone and Pituitary Program (National Institutes of Health, Bethesda, MD, U.S.A.).

Cell culture

Rat thyroid FRTL-5 cells were originally obtained from the Interthyr Research Foundation (Bethesda, MD, U.S.A). The cells were grown in Coon's modified Ham's F12 medium, supplemented with 5 % (v/v) calf serum and six hormones [34] (insulin, 1 μg/ml; transferrin, 5 μg/ml; cortisol, 10 nM; the tripeptide Gly-L-His-L-Lys, 10 ng/ml; TSH, 0.3 m-unit/ml; somatostatin, 10 ng/ml) in a water-saturated atmosphere of air/CO_2 (19:1) at 37 °C. Before an experiment, cells from one donor culture dish were harvested with a 0.1 % trypsin solution and plated on to plastic 100-mm culture dishes. The cells were grown for 7–8 days before an experiment, with 2–3 changes of the culture medium. Fresh medium was always added 24 h prior to an experiment. For the [^{3}H]phorbol 12,13-dibutyrate binding assay the cells were grown on microtitration plates, and were used when cells reached confluency (3–4 days after plating).

Measurement of $[Ca^{2+}]_i$

The medium was aspirated, and the cells were harvested with Hepes-buffered saline solution (HBSS; 118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 1 mM $CaCl_2$ and 20 mM Hepes, pH 7.2) lacking calcium but containing 0.02 % EDTA and 0.1 % trypsin. After washing the cells three times with HBSS by pelleting, the cells were incubated with 1 μM fura 2/AM for 30 min at 37 °C. Following the loading period, the cells were washed twice with HBSS buffer, and incubated for at least 10 min at room temperature, and washed once again. Fluorescence was measured with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM $CaCl_2$ and Triton X-100 to obtain maximal fluorescence. Chelation of extracellular Ca^{2+} with 5 mM EGTA and the addition of Tris-base were used to elevate pH above 8.3, to obtain minimal fluorescence. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [35], using a computer program designed for the fluorimeter and a K_d value of 224 nM for fura 2. In some experiments, the cells were kept in sodium-free HBSS. In this buffer, NaCl was exchanged with equimolar concentrations of choline chloride. Furthermore, in some experiments we measured the calcium extrusion by calculating the change in $[Ca^{2+}]_i$ of the downward slope of the thapsigargin-evoked change in $[Ca^{2+}]_i$. In these experiments, a line was drawn and the maximal rate of change in $[Ca^{2+}]_i$ was calculated as $\Delta[Ca^{2+}]_i$ over a period of 10 s.

Measurement of extracellular free calcium concentration ($[Ca^{2+}]_o$)

The cells were grown and harvested as for the $[Ca^{2+}]_i$ experiments. The harvested cells were allowed to stabilize in a water-saturated atmosphere of air/CO_2 (19:1) at 37 °C for 2 h. The experiment was performed as described previously [36]. In brief, the cells were washed and resuspended in calcium-free HBSS in a quartz cuvette. Prior to an experiment, fluo 3 pentapotassium salt (final concentration 1 μM) was added to the cuvette. EGTA was then added to buffer $[Ca^{2+}]_o$ to 100–200 nM. Next, the cells were stimulated with vehicle or H_2O_2 (final concentration 300 μM) for 1 min, and then thapsigargin (1 μM) or DMSO was added, and the changes in $[Ca^{2+}]_o$ were measured. Fluorescence was measured with a Hitachi F2000 fluorimeter. The excitation wavelength was 480 nm and emission was measured at 530 nm. The signal was calibrated by addition of 1 mM $CaCl_2$ and Triton X-100 to obtain maximal fluorescence. Chelation of extracellular Ca^{2+} with 5 mM EGTA and the addition of Tris-base were used to elevate pH above 8.3, to obtain minimal fluorescence. $[Ca^{2+}]_i$ was calculated using a K_d value of 370 nM for fluo 3.

[^{3}H]Phorbol 12,13-dibutyrate binding assay

The assay was performed as described by Trilivas and Brown [37] with certain modifications. Briefly, the medium was aspirated and the cells were pretreated with vehicle or thapsigargin (final concentration 2 μM) in medium, and the cells were incubated for 90 s. Subsequently vehicle or H_2O_2 was added for 60 s, and the cells were washed three times with ice-cold HBSS. [^{3}H]Phorbol 12,13-dibutyrate (final concentration 10 nM) either with (non-specific binding) or without (total binding) PMA (final concentration 1 μM) was added together with the test compounds. After washing the cells with HBSS, the radioactivity was measured using a Wallac Microbeta counter. The results are shown as specific binding of [^{3}H]phorbol 12,13-dibutyrate per well.

PKC extraction and assay

Rat brain PKC was extracted and assayed as previously described [38], with some modifications. Briefly, rat brain was homogenized in ice-cold calcium-free 10 mM Hepes-buffer (pH 7.5) containing 100 μM leupeptin and 1 mM PMSF. Crude particles were removed by spinning the suspension at 200 ***g*** (for 10 min at 4 °C). The supernatant was further centrifuged (100000 ***g***, for 60 min at 4 °C). The resulting supernatant was partially purified by DEAE-Sephadex (Pharmacia, Sweden) column chromatography. The protein contents in the fractions were measured according to Bradford [39]. The fraction showing the highest

calcium- and phospholipid-dependent kinase activity was stored in 50 % (v/v) glycerol at −20 °C.

Before the assay, H_2O_2 and the PKC protein extract were mixed and incubated for 15 min. The assay was started by adding the substrate to the mixture. The final reaction mixture (7 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mM EGTA, 0.08 mM substrate peptide Arg-Lys-Gly-Ser-Leu-Arg-Gly-NH_2 and 10 mM Hepes, pH 7.5) contained, in a final volume of 100 μl, 1 μg of PKC protein extract and 100 μM [^{32}P]ATP, with (total activity) or without (non-specific activity) 1 mM $CaCl_2$, phosphatidylserine (40 μg/ml) and 1,2-*sn*-dioctanoylglycerol (8 μg/ml). The reaction was stopped by spotting the reaction mixture on to Whatman P81 phosphocellulose paper strips and washing the strips in 75 mM phosphoric acid. The trapped radioactivity was counted after air-drying. PKC activity was calculated by subtracting the non-specific activity from the total activity. PKC activity was calculated as nmol of ATP transferred to substrate/min per mg of protein extract. The obtained kinase activities were plotted semi-logarithmically, and EC_{50}-values were calculated (nonlinear regression; GraphPad Prism 2.0b for Macintosh).

Western blot of PKC isoenzymes

FRTL-5 cells were grown and harvested as for the $[Ca^{2+}]_i$ experiments. The cells were detached from culture plates and washed three times with HBSS. After 30 min at 37 °C in HBSS, cells were treated with 300 μM H_2O_2 in HBSS for 3 or 15 min at 37 °C. Cytosolic and particulate fractions were prepared as described by Kass et al. [40]. The protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). The samples were then quick-frozen in liquid nitrogen and stored at −70 °C.

Cytosolic and particulate fractions (15 μg of protein/sample) were subjected to SDS/PAGE (10 % polyacrylamide) for PKC analysis. The proteins where transferred on to nitrocellulose membrane (Schleicher & Schuell) by wet-blotting (Hoefer). Western blot analysis was performed using isoenzyme-specific PKC antibodies. Mouse monoclonal antibodies raised against PKCα, PKCδ and PKCε were obtained from Transduction Laboratories (Lexington, KY, U.S.A.), and rabbit polyclonal antibody raised against PKCζ was from Boehringer Mannheim. The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Sigma). The proteins were detected by enhanced chemiluminescence (ECL®). Densitometric analysis was performed using the PhotoMagic and Image Gauge programs.

Measurement of cellular ATP

Cells were grown and harvested as for the $[Ca^{2+}]_i$ experiments, and were allowed to rest for 30 min at 37 °C. Aliquots of cells were then stimulated with vehicle or 300 μM H_2O_2 for the times indicated. In some experiments, cells were incubated in the presence of 10 mM 2-deoxy-D-glucose and 10 μM oligomycin. The incubation was stopped by the addition of trichloroacetic acid (final concentration 0.2 %) and EGTA (final concentration 2 mM), and the cellular ATP content was measured using a commercial ATP-assay kit (BioOrbit, Turku, Finland).

Measurement of membrane potential using bisoxonol

The cells were grown and harvested as for the calcium experiments. After the final wash, the cells were added to a quartz cuvette, and bisoxonol (final concentration 100 nM) was added. The cells were equilibrated with bisoxonol for at least 15 min prior to an experiment. The cells were stimulated with H_2O_2 or vehicle. The excitation wavelength was 540 nm, and the emission wavelength was 580 nm [41]. Each experiment was calibrated by the addition of 50 mM KCl, in order to make comparison between different experiments possible. In some experiments the cells had been pretreated with 2 μM PMA for 24 h to down-regulate PKC.

Electrophysiological measurements of membrane potential

Electrophysiological recordings were performed using an EPC-9 amplifier and 'Pulse/PulseFit' software (HEKA, Lambrecht, Germany). The pipettes had a resistance of 5–6 MΩ when filled with intracellular solution (140 mM KCl, 1 mM $CaCl_2$, 11 mM EGTA and 10 mM Hepes, pH 7.3; calculated free Ca^{2+} concentration less than 100 nM). Cells grown on small round coverslips were transferred to the experimental chamber (volume approx. 500 μl) immediately before use and washed extensively with extracellular medium (Hepes-buffered Dulbecco's modified saline solution containing 136 mM NaCl, 2.6 mM KCl, 1.46 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 20 mM Hepes, pH 7.4). After formation of the whole-clamp configuration [42], the amplifier was switched to 'slow' current-clamp mode and the recordings were initiated approx. 2 min after stabilization of a cell membrane potential. Test compounds were applied to the bath using a peristaltic pump (perfusion rate 3 ml/min). *Post hoc* statistical analysis and graphic presentation were performed using Microsoft Excel Software. In some experiments the cells had been pretreated with 2 μM PMA for 24 h to down-regulate PKC.

Statistics

The results are expressed as means ± S.E.M. Statistical analysis was carried out using Student's *t* test for paired observations. When three or more means were tested, analysis of variance was used. The calcium traces shown are representative of at least four separate experiments from at least two batches of cells. The traces shown in the Figures are usually from the same batch of cells. The [^{3}H]phorbol 12,13-dibutyrate binding data were analysed for homogeneity of group variances using Bartlett's test. For analysing parametric data, analysis of variance and Tukey's test were used. For non-parametric data, Kruskal-Wallis analysis followed by the Mann–Whitney *U* test and the Dunn–Sidak correction were performed.

RESULTS

H_2O_2 attenuates calcium entry

We first investigated whether exogenous H_2O_2 could modify $[Ca^{2+}]_i$ in FRTL-5 cells. In control cells, thapsigargin evoked a rapid increase in $[Ca^{2+}]_i$ (275 ± 17 nM, $n = 6$), which then stabilized at a new elevated plateau level, 223 ± 22 nM above the pre-stimulatory level. The thapsigargin-evoked plateau level is the result of store-operated calcium entry [33]. In cells treated with 300 μM H_2O_2 the initial thapsigargin-evoked increase in $[Ca^{2+}]_i$ was 266 ± 12 nM ($n = 6$), whereas the new elevated $[Ca^{2+}]_i$ level was 138 ± 20 nM ($P < 0.05$ compared with control cells; Figure 1A). H_2O_2 attenuated the entry of calcium in a concentration-dependent manner both in a calcium buffer (Figures 1A and 1B), and when calcium was re-added to cells stimulated with thapsigargin in a calcium-free buffer (Figures 1C and 1D). This effect of H_2O_2 was abrogated by pretreatment of the cells with 1 mM β-mercaptoethanol (Figure 1E). H_2O_2 itself was without a significant effect on basal $[Ca^{2+}]_i$, although a small decrease in the basal level of $[Ca^{2+}]_i$ was observed in some

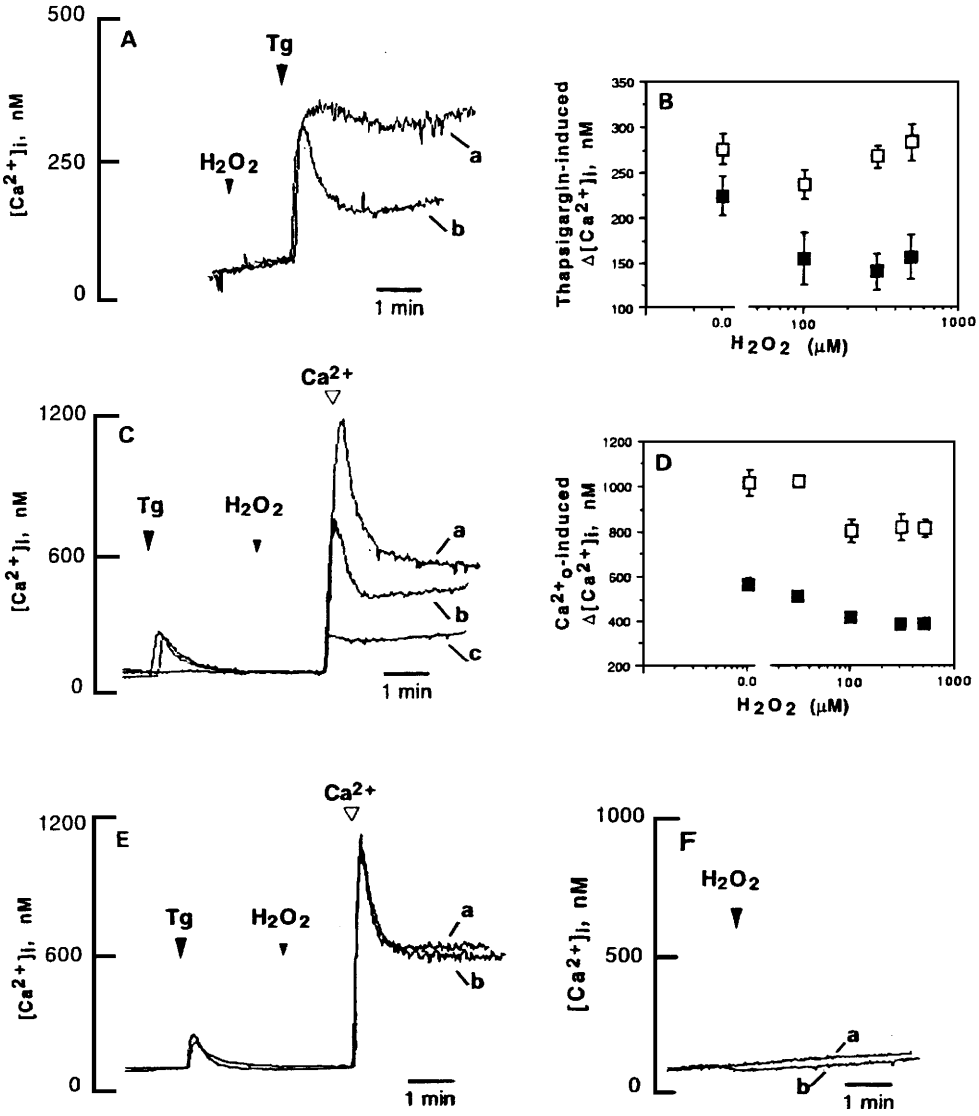

Figure 1 H_2O_2 inhibits calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (**A**) Vehicle (a) or 300 μM H_2O_2 (b) was added to the cells, which were then stimulated with 1 μM thapsigargin (Tg) in a calcium-containing buffer. (**B**) Dose-dependent effect of H_2O_2 on the transient increase in $[Ca^{2+}]_i$ (□) and the new plateau level of $[Ca^{2+}]_i$ (■), in cells stimulated with thapsigargin. $\Delta[Ca^{2+}]_i$ gives the change in $[Ca^{2+}]_i$ between the pre-stimulatory level of $[Ca^{2+}]_i$ and either the transient level or the new plateau level of $[Ca^{2+}]_i$. Each point represents the mean ± S.E.M. of four to seven determinations. (**C**) The cells were stimulated with 1 μM thapsigargin in a calcium-free buffer containing 100 μM EGTA. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by calcium (final concentration 1 mM). Control cells were not stimulated with thapsigargin (c). (**D**) Dose-dependent effect of H_2O_2 on the transient increase in $[Ca^{2+}]_i$ (□) and the new plateau level of $[Ca^{2+}]_i$ (■) when calcium (final concentration 1 mM) was added to cells stimulated with thapsigargin. Each point represents the mean ± S.E.M. of four to seven determinations. (**E**) The cells were stimulated with 1 μM thapsigargin in a calcium-free buffer containing 100 μM EGTA and 1 mM β-mercaptoethanol. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by calcium (final concentration 1 mM). (**F**) Cells in a calcium-containing buffer were stimulated with vehicle (a) or 300 μM H_2O_2 (b). The traces shown are representative of at least four separate experiments.

experiments (Figure 1F). In a calcium-free buffer, 300 μM H_2O_2 itself did not evoke a change in $[Ca^{2+}]_i$ during a 6-min incubation (results not shown). The attenuating effect of H_2O_2 was also evident in a sodium-free buffer (Figure 2), suggesting that H_2O_2 did not enhance Na^+/Ca^{2+} exchange. Furthermore, we observed that H_2O_2 abrogated the thapsigargin-evoked entry of barium (Figure 3A). As barium is a very poor substrate for the Ca^{2+}-ATPase [43], it cannot be extruded from the cells or sequestered in the cells. We also observed that the manganese-evoked quenching of fura 2 was attenuated in cells treated with H_2O_2, compared with control cells (Figure 3B). Thus our results suggest that H_2O_2 attenuates the entry of calcium. A summary of the experiments is given in Table 1.

Mechanism of action of H_2O_2

Oxidizing agents, like H_2O_2, may activate PKC [30,31], and activation of PKC attenuates calcium entry in FRTL-5 cells [33]. In cells pretreated for 24 h with 2 μM of the phorbol ester PMA, which results in down-regulation of the α, δ and ϵ isoenzymes of PKC in FRTL-5 cells [44], the inhibitory effect of 300 μM H_2O_2

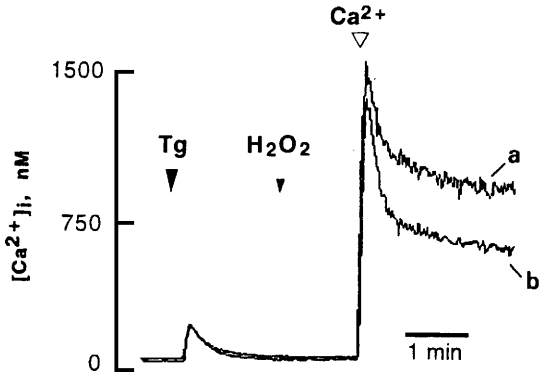

Figure 2 H_2O_2 inhibits calcium entry in a sodium-free buffer

The cells were harvested and loaded with fura 2 as described in the Materials and methods section.The cells were stimulated with 1 μM thapsigargin (Tg) in a sodium-free, calcium-free, choline chloride buffer containing 100 μM EGTA. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by calcium (final concentration 1 mM). The experiments were repeated at least four times.

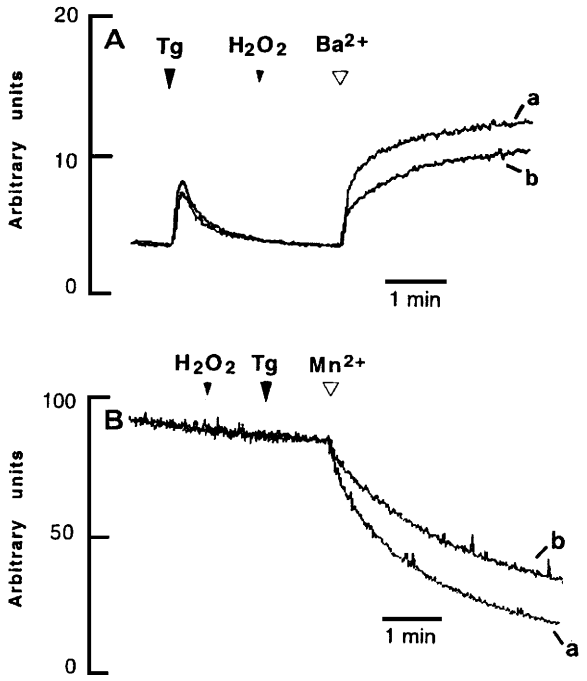

Figure 3 H_2O_2 inhibits barium and manganese entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (**A**) The cells were stimulated with 1 μM thapsigargin (Tg) in a calcium-free buffer containing 100 μM EGTA. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by barium (final concentration 1 mM). (**B**) The cells were stimulated with 1 μM thapsigargin (Tg) in a calcium buffer. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by manganese (final concentration 0.1 mM). The experiments were repeated at least four times.

was totally abolished (Figure 4A). In cells treated with the kinase inhibitor staurosporine (200 nM for 10 min), the effect of 300 μM H_2O_2 on the thapsigargin-evoked entry of calcium was again totally abolished (Figure 4B and see Table 1). Furthermore, in cells treated with the PKC inhibitor calphostin C (100 nM for 10 min), H_2O_2 was without an effect on store-operated calcium entry (Table 1). The results therefore suggest that H_2O_2 attenuates

Table 1 Effect of H_2O_2 on $[Ca^{2+}]_i$

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. The cells were first stimulated with 1 μM thapsigargin in a calcium-free buffer containing 100 μM EGTA, and then 1 mM calcium was re-added to the cell suspension and the changes in $[Ca^{2+}]_i$ were measured (Control). Alternatively the cells were treated with 300 μM H_2O_2 after the addition of thapsigargin but prior to the addition of calcium (H_2O_2). In some experiments, the cells were resuspended in a sodium-free buffer (NaCl was substituted with equimolar concentrations of choline chloride), or the experiments were performed in the presence of 1 mM β-mercaptoethanol (βMKEtOH), or the cells were pretreated with 200 nM staurosporin for 10 min. In some experiments, the cells were pretreated with 2 μM PMA for 24 h to down-regulate PKC (PKC d-r) or with 100 nM calphostin C for 10 min. The values represent the mean $\pm$ S.E.M. of three to eight separate experiments. $^*P < 0.05$ compared with control cells.

	$\Delta[Ca^{2+}]_i$ (nM)			
	Control		H_2O_2	
	Peak	Plateau	Peak	Plateau
No treatment	1013 $\pm$ 60	563 $\pm$ 29	813 $\pm$ 57*	381 $\pm$ 20*
Sodium-free buffer	1227 $\pm$ 64	778 $\pm$ 31	1084 $\pm$ 69	477 $\pm$ 41*
β-MKEtOH	1172 $\pm$ 45	691 $\pm$ 51	1271 $\pm$ 19	652 $\pm$ 35
Staurosporine	1307 $\pm$ 90	962 $\pm$ 96	1382 $\pm$ 95	880 $\pm$ 23
PKC d-r	1513 $\pm$ 70	856 $\pm$ 33	1544 $\pm$ 20	819 $\pm$ 23
Calphostin C	806 $\pm$ 54	264 $\pm$ 25	773 $\pm$ 72	377 $\pm$ 52

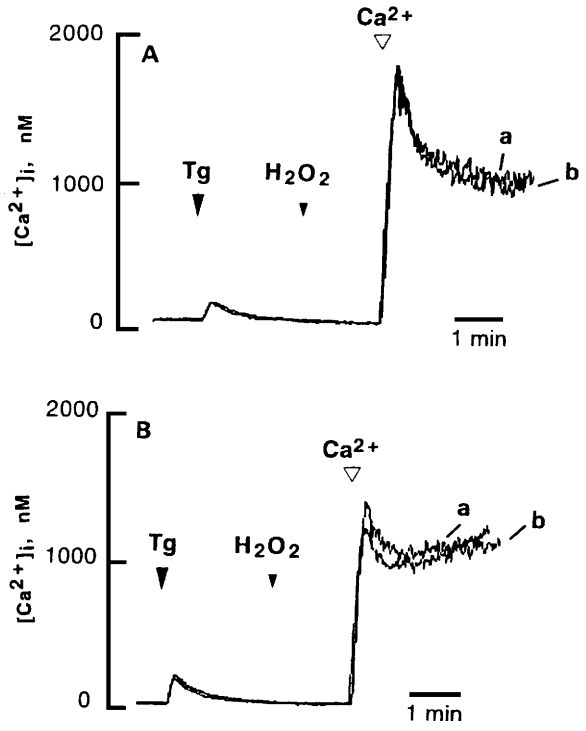

Figure 4 Inhibition of PKC abrogates the effect of H_2O_2 on calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (A) The cells were pretreated with 2 μM PMA for 24 h. The cells were then stimulated with 1 μM thapsigargin (Tg) in a calcium-free buffer containing 100 μM EGTA. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by calcium (final concentration 1 mM). (**B**) The cells were pretreated with 200 nM staurosporine for 10 min. The cells were stimulated with 1 μM thapsigargin in a calcium-free buffer containing 100 μM EGTA. Vehicle (a) or 300 μM H_2O_2 (b) was then added, followed by calcium (final concentration 1 mM). The traces shown are representative of at least four separate experiments.

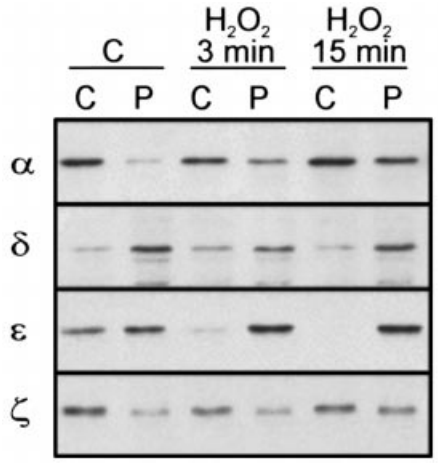

Figure 5 Translocation of PKC isoenzymes after stimulation with H_2O_2

(**A**) The cells were stimulated with H_2O_2 and fractionated as described in the Materials and methods section. The blots show the effect of 300 μM H_2O_2 on the distribution of PKCα (α), PKCδ (δ), PKCϵ (ϵ) and PKCζ (ζ) between the cytosolic (C) and particulate (P) fractions after a 3- and a 15-min stimulation. Each experiment was performed three times.

store-operated calcium entry by a mechanism dependent on activation of PKC.

Effect of H_2O_2 on activation of PKC in FRTL-5 cells

To test the effect of H_2O_2 on PKC activity, we measured the binding of [^{3}H]phorbol 12,13-dibutyrate to PKC. H_2O_2 (100 μM) induced a weak ($16 \pm 4.5\,\%$), but significant ($P < 0.05$) increase in the binding of [^{3}H]phorbol 12,13-dibutyrate, compared with control experiments. Higher concentrations did not show an enhanced effect. In line with previous studies [45], we observed that H_2O_2 slightly attenuated PKC activation in a partially purified preparation of PKC obtained from rat brain. The basal PKC activity was 149 nmol/mg per min. Addition of H_2O_2 (10 μM to 10 mM) reduced the specific PKC activity in a concentration-dependent manner. The EC_{50} for H_2O_2 was 1540 μM (two independent assays with two parallel determinations). This suggests that the effect of H_2O_2 on PKC in intact cells is probably an indirect effect.

Effects of H_2O_2 on various isoenzymes of PKC in FRTL-5 cells

Using Western blot analysis, we showed that stimulating the cells with 300 μM H_2O_2 resulted in a rapid and significant ($P < 0.05$) translocation of the PMA-sensitive isoenzyme PKCϵ from the cytosolic fraction to the particulate fraction (Figure 5). Densitometric analysis showed that the relative distribution of PKCϵ in control cells was $39 \pm 4\,\%$ and $61 \pm 4\,\%$ in the cytosolic and the particulate fractions respectively. After 3 min of stimulation with 300 μM H_2O_2, the relative distribution of PKCϵ was $22 \pm 5\,\%$ and $78 \pm 5\,\%$, and after 15 min $17 \pm 4\,\%$ and $83 \pm 4\,\%$ in the cytosolic and the particulate fractions respectively. However, H_2O_2 was without a significant effect on the distribution of the PKCα, PKCδ and PKCζ isoenzymes (results not shown). Our results therefore support our conclusion that H_2O_2 attenuates the thapsigargin-evoked increase in $[Ca^{2+}]_i$ by mechanisms that are dependent on the activation of PKC.

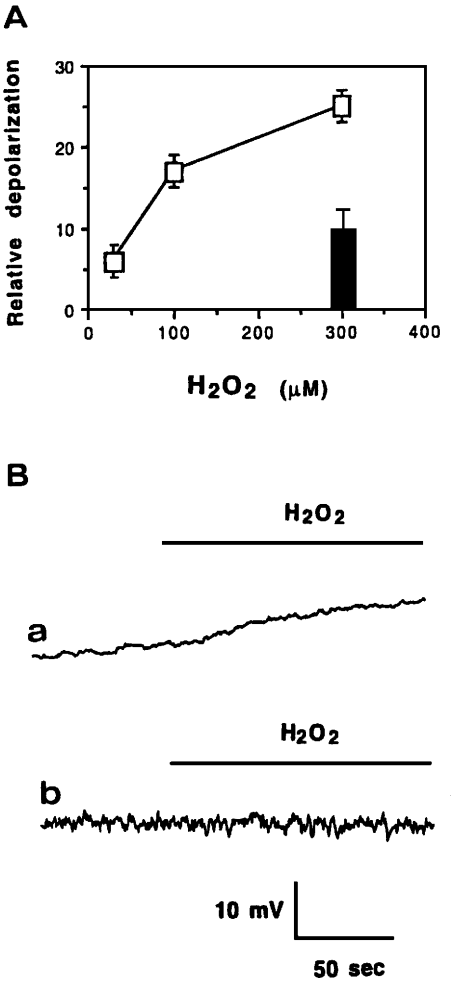

Figure 6 H_2O_2 depolarizes the membrane potential

(**A**) The cells were harvested and loaded with bisoxonol as described in the Materials and methods section. The dose-dependent effect of H_2O_2 on membrane potential was determined. The bar shows the H_2O_2-evoked depolarization obtained with cells pretreated with 1 μM PMA for 24 h to down-regulate PKC. At the end of an experiment, the cells were depolarized by the addition of 50 mM potassium chloride, to make a comparison between different experiments possible. The results are given as the percentage depolarization of that obtained with 50 mM potassium chloride. Each point represents the mean $\pm$ S.E.M. of three to four separate experiments. (**B**) Effect of H_2O_2 on current-clamped cells. Trace a, the cells were perfused with 300 μM H_2O_2, and the change in membrane potential was recorded. Trace b, the cells were pretreated with 2 μM PMA for 24 h, and then the effect of 300 μM H_2O_2 on membrane potential was tested on current-clamped cells. The traces are representative recordings from three to five experiments.

Effect of H_2O_2 on membrane potential

In a previous study we showed that activation of PKC depolarized FRTL-5 cells, and thus decreased calcium entry due to a decreased electrochemical gradient for calcium [33]. In the present study we showed that H_2O_2 depolarized the plasma membrane in a concentration-dependent manner (Figure 6A). In cells treated with 2 μM PMA for 24 h to down-regulate PKC, the depolarizing effect of H_2O_2 was significantly ($P < 0.05$) attenuated (Figure 6A).

We next investigated the effect of H_2O_2 on the membrane potential using patch-clamp in the whole-cell mode [42]. Current-clamp experiments showed that the mean resting membrane potential was 39.5 ± 3.7 mV. Addition of H_2O_2 (final concen-

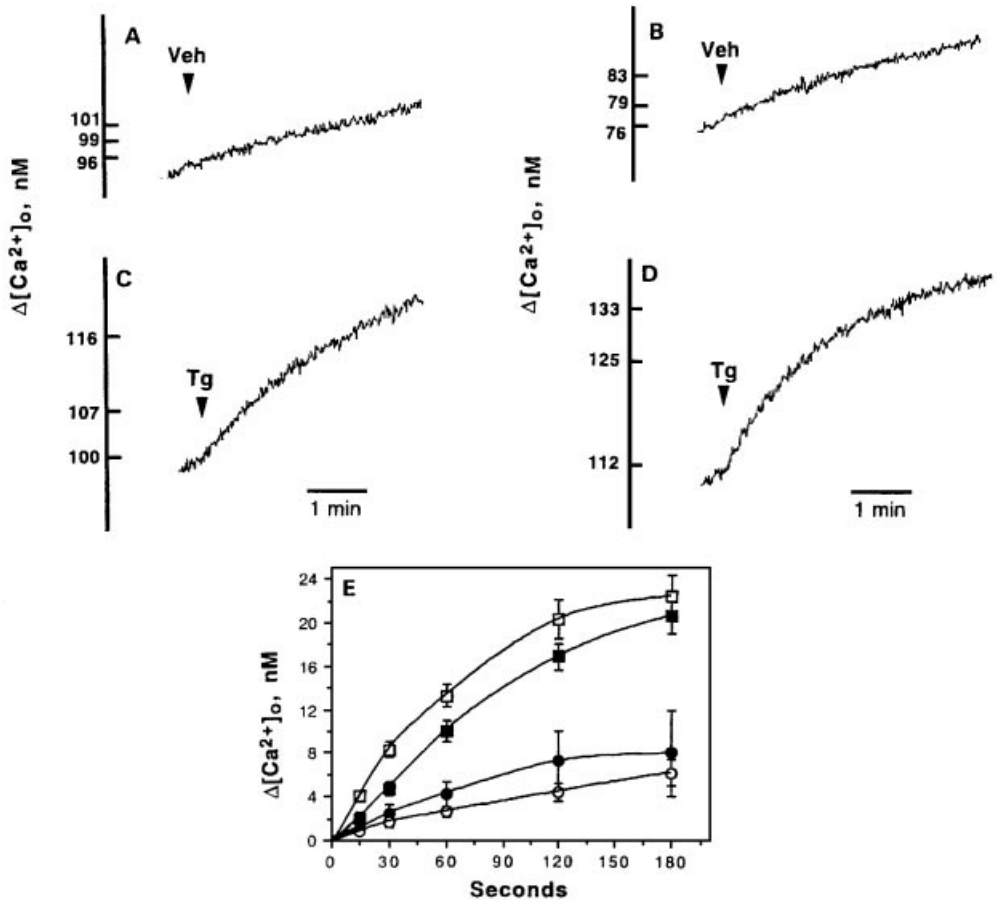

Figure 7 H_2O_2 enhances calcium efflux in FRTL-5 cells

The cells were incubated in a nominally calcium-free buffer in the presence of fluo 3. The extracellular calcium was then adjusted with EGTA to 100–200 nM and the cells were stimulated with vehicle (Veh) or thapsigargin (Tg). (**A**) Control cells. (**B**) Cells treated with 300 μM H_2O_2. (**C**) Control cells stimulated with 1 μM thapsigargin. (**D**) Cells treated with 300 μM H_2O_2 and then stimulated with 1 μM thapsigargin. (**E**) Summary of all the separate experiments described in (**A**)–(**D**). Control cells (●), cells treated with 300 μM H_2O_2 (○), control cells stimulated with 1 μM thapsigargin (■) and cells treated with 300 μM H_2O_2 and then stimulated with 1 μM thapsigargin (□). Each point represents the mean ± S.E.M. of six to eight separate determinations.

tration 300 μM) decreased the membrane potential to 31.4 ± 3.0 mV (ΔV_m 8.2 ± 1.6 mV, Figure 6B). In cells treated with 2 μM PMA for 24 h to down-regulate PKC, H_2O_2 was without an effect. In these cells the resting potential was 37.9 ± 3.6 mV and after addition of H_2O_2 36.2 ± 3.7 mV (ΔV_m 2.0 ± 0.5 mV, $P < 0.05$ compared with the depolarization observed in cells not treated with PMA).

In cells depolarized with 10 mM KCl, which depolarizes the cells to the same extent as does 300 μM H_2O_2 (as measured with bisoxonol), the addition of calcium (final concentration 1 mM) to cells stimulated with 1 μM thapsigargin in a calcium-free buffer, increased $[Ca^{2+}]_i$ transiently by 774 ± 17 nM, which then stabilized at a new pleateu level, 432 ± 9 nM above the pre-stimulatory level of $[Ca^{2+}]_i$. In control cells the transient increase in $[Ca^{2+}]_i$ was 971 ± 41 nM ($P < 0.05$) and the new plateau level was 513 ± 25 nM ($P < 0.05$). Thus a relatively modest depolarization can significantly decrease store-operated calcium entry.

H_2O_2 enhances calcium extrusion in FRTL-5 cells

The effect of H_2O_2 on membrane potential was modest, and may only in part explain the attenuated increase in $[Ca^{2+}]_i$ after stimulation with thapsigargin. Thus we measured the thapsigargin-induced extrusion of calcium from cells treated with H_2O_2. We observed that the initial extrusion of calcium (4 ± 0.4 nM/15 s) after addition of thapsigargin (final concentration 1 μM) was significantly ($P < 0.05$) enhanced, compared with vehicle-treated cells (2 ± 0.5 nM/15 s; Figures 7C and 7D). H_2O_2 itself was without an effect, compared with vehicle (1 ± 0.2 nM/15 s and 1 ± 0.3 nM/15 s, respectively; Figures 7A and 7B). A summary of the results is shown in Figure 7(E). A complete abrogation of the enhancing effect of H_2O_2 on the thapsigargin-evoked extrusion of calcium was observed in cells treated with β-mercaptoethanol (3 ± 0.6 nM/15 s, $n = 5$ and 2 ± 0.6 nM/15 s, $n = 6$, in control and H_2O_2-treated cells respectively), as well as in cells with down-regulated PKC (4 ± 0.5 nM/15 s, $n = 4$ and 4 ± 0.7 nM/15 s, $n = 5$, in control and H_2O_2-treated cells respectively). Neither β-mercaptoethanol nor down-regulation of PKC had any effects on the basal calcium extrusion (i.e. in cells not stimulated with thapsigargin; results not shown).

Furthermore, when cells in a calcium-free buffer were first stimulated with 300 μM H_2O_2 and then with thapsigargin, a difference in the downward slope of the thapsigargin-evoked change in $[Ca^{2+}]_i$ was observed (i.e. in the rate of calcium

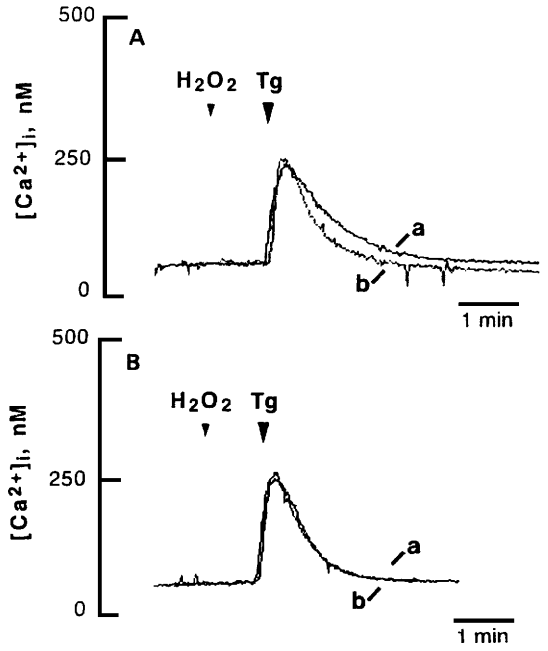

Figure 8 H_2O_2 enhances calcium extrusion in FRTL-5 cells

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (**A**) The cells were treated with vehicle (a) or 300 μM H_2O_2 (b) and then stimulated with 1 μM thapsigargin (Tg) in a calcium-free buffer containing 100 μM EGTA, and the rate of calcium extrusion (i.e. the downward slope of $[Ca^{2+}]_i$) was calculated (see the Results section). (**B**) The cells were pretreated with 2 μM PMA for 24 h and were then treated with vehicle (a) or 300 μM H_2O_2 (b) and finally stimulated with 1 μM thapsigargin (Tg) in a calcium-free buffer containing 100 μM EGTA, and the rate of calcium extrusion was calculated. The traces shown are representative recordings from seven to eight separate experiments.

extrusion from the cells; Figure 8A). In cells treated with H_2O_2 the decrease in $[Ca^{2+}]_i$ was 33 ± 2.9 nM/10 s, whereas in control cells the change was 22 ± 1.3 nM/10 s ($P < 0.05$). In PKC-down-regulated cells, no significant difference was observed in H_2O_2-treated cells (29 ± 3.3 nM/10 s), compared with control cells (26 ± 3.1 nM/10 s; Figure 8B).

Effect of H_2O_2 on cellular ATP

Oxidizing compounds may deplete the cellular ATP content, and thus interfere with several processes. In FRTL-5 cells treated with 300 μM H_2O_2 for 3 min, the cellular ATP content was $74 \pm 12\%$ ($P < 0.05$, compared with control; results not shown). Incubation of the cells for 15 min with this concentration of H_2O_2 decreased the cellular ATP content to $11 \pm 5\%$ of that seen in control cells, and a 15-min incubation of the cells in the presence of 10 mM 2-deoxy-D-glucose and 10 μM oligomycin almost totally depleted cellular ATP ($0.8 \pm 0.1\%$ of that seen in control cells; results not shown).

DISCUSSION

The present results suggest a novel role for H_2O_2 in thyroid cells, i.e. an attenuation of store-operated calcium entry and the concomitant increase in $[Ca^{2+}]_i$, which may be of physiological importance. We base our conclusion on the following observations. First, H_2O_2 clearly inhibited the thapsigargin-evoked plateau level in $[Ca^{2+}]_i$. Second, H_2O_2 inhibited the thapsigargin-evoked entry of both manganese and barium in the cells. Manganese and barium enter the cytosol from the extracellular space through the same pathways as calcium. Thus if H_2O_2 attenuates store-operated calcium entry, the entry of both manganese and barium should also be decreased, as was also observed in our experiments. Interestingly, the initial rate of increase in $[Ca^{2+}]_i$ evoked by re-addition of calcium to cells stimulated with thapsigargin was not affected by H_2O_2 (see Figure 1D). Presently we do not have an explanation for this observation. However, it is possible that the initial entry of calcium is necessary to trigger the H_2O_2-evoked attenuation of store-operated calcium entry.

In thyroid cells, including FRTL-5 cells, calcium-mediated mechanisms are of crucial importance in regulating thyroid hormone synthesis [7–9]. The activity of a calcium/NADPH-dependent thyroid peroxidase in the plasma membrane is considered to be the limiting step in the iodination of tyrosine groups and the production of thyroid hormones. Thus agonists which increase $[Ca^{2+}]_i$ potently activate this system. However, excessive production of H_2O_2 may be detrimental to the cells. In other cell types, H_2O_2 blocks the endoplasmic Ca^{2+}-ATPase [46], releases calcium from mitochondria [47], interferes with activation of transcription factors [19], and may initiate apoptosis [20]. The results in the present study suggest that a negative-feedback inhibition of H_2O_2 production, evoked by H_2O_2 itself, exists in thyroid cells. This inhibition is the result of both the H_2O_2-evoked inhibition of calcium entry and the H_2O_2-evoked efflux of calcium from the cells. The sum of these effects would then be a decreased calcium-dependent production of H_2O_2. The mechanism may also be of importance in the fine-tuning of the production of thyroid hormones.

Activation of PKC decreases store-operated calcium entry in several cell types [48,49], including FRTL-5 cells [33]. At least in HEL cells and in Jurkat T-cells, activation of the PKCβ isoenzyme specifically abrogated calcium entry [50]. We obtained a small, but significant increase in the binding of [^{3}H]phorbol 12,13-dibutyrate in response to H_2O_2 and a clear translocation of the PKCϵ isoenzyme from the cytosolic to the particulate fraction. Our results suggest that the effects of H_2O_2 on calcium entry may be mediated via activation of PKCϵ, as the effect of H_2O_2 was inhibited by staurosporine and calphostin C, and in cells with down-regulated PKC. It has recently been shown that lengthy treatment of FRTL-5 cells with PMA down-regulates the α, δ and the ϵ isoenzymes, but not the ζ isoenzyme of PKC [44]. Although the increased binding of [^{3}H]phorbol 12,13-dibutyrate is not a direct measure of PKC activity, it correlates with the *in situ* activity [37]. It is, of course, impossible to exclude the possibility that staurosporine, calphostin C or down-regulation of PKC with PMA attenuated the effect of H_2O_2 on calcium entry via some PKC-independent mechanisms. However, the present results strongly suggest a role for H_2O_2 that is mediated via activation of PKC. Several other studies have shown that H_2O_2 activates PKC in intact cells [30,31], whereas H_2O_2 may inactivate purified preparations of PKC [45]. We also observed that H_2O_2 attenuated PKC activity in a cell-free preparation, suggesting that the effect of H_2O_2 on PKC activity in intact cells is indirect.

Our previous results showed that activation of PKC resulted in a depolarization of the membrane potential [33]. This decreases the electrochemical gradient for calcium, resulting in a decreased influx of calcium. In the present study we clearly show that H_2O_2 depolarizes the cell membrane, and that this effect is attenuated in cells with down-regulated PKC. This result further supports our conclusion, that the effect of H_2O_2 is mediated via PKCϵ. In addition, the magnitude of the depolarization is sufficient to attenuate calcium entry, as a depolarization of the same magnitude as that seen with H_2O_2, but evoked with a low concentration of KCl, also attenuated calcium entry. Presently we

do not know by which mechanism(s) PKC depolarizes the membrane potential in our cells. It is quite possible that PKC may inactivate potassium channels in the plasma membrane as has recently been reported [51]. In addition, we also observed that H_2O_2 enhanced the extrusion of calcium from cells stimulated with thapsigargin. This effect was also inhibited in cells with down-regulated PKC. Presently we do not know by which mechanism(s) the H_2O_2-evoked extrusion of calcium is mediated. Several G-protein- and tyrosine kinase-coupled receptors may activate calcium extrusion by activating a plasma membrane Ca^{2+}-ATPase [52,53]. We have previously shown that H_2O_2 does not enhance plasma membrane Ca^{2+}-ATPase activity [28]. However, although the antibody used for immunoprecipitation in those experiments recognizes all the presently known Ca^{2+}-ATPases, we cannot exclude the possibility that H_2O_2 activates some other, presently unknown Ca^{2+}-ATPase (or other calcium extrusion mechanism). Based on the findings in the present study, this possibility seems very likely. The small decrease in the basal $[Ca^{2+}]_i$ level seen after the addition of H_2O_2 in some experiments could also be the result of such an activation of calcium extrusion. This result also clearly shows that H_2O_2 does not release calcium from intracellular stores, although the cellular ATP content is decreased. Furthermore, as the calcium/NADPH-dependent thyroid peroxidase is strictly localized to the plasma membrane in thyroid cells, the produced H_2O_2 may efficiently modulate the function of calcium and potassium channels and calcium extrusion mechanisms. A role for PKC in this modulation is strengthened by the fact that a substantial amount of the cellular PKC is already localized to the plasma membrane in unstimulated cells, where it can rapidly interact with membrane proteins upon stimulation.

In thyroid cells, some toxic effects of H_2O_2 have been reported [12]. In these experiments the cells had to be exposed to a high concentration of H_2O_2 (5 mM) for 1 h. Furthermore, recent results have shown that in pig thyroid cells, incubated with H_2O_2 (up to 300 μM) for 1 h, only 1 % of the cells showed signs of damage [14]. It is well known that thyroid cells have a remarkable capacity to degrade H_2O_2 [54], and the fact that all the observed effects of H_2O_2 could be abolished when PKC was inhibited strongly suggests that our results are not due to a toxic effect of H_2O_2.

In conclusion, the results obtained in the present study define a new mechanism of action of H_2O_2 in FRTL-5 cells, i.e. an attenuation of store-operated calcium entry. This mechanism appears to be mediated via activation of PKC. The present observation is especially important if it also occurs in thyroid cells other than FRTL-5 cells, as the agonist-evoked production of H_2O_2 is calcium-dependent in thyroid cells. In addition to participating in the production of thyroid hormones, the produced H_2O_2 may also exert an inhibitory effect on agonist-evoked increases in $[Ca^{2+}]_i$, and thus regulate its own production.

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