Redox modulation of intracellular free calcium concentration in thyroid FRTL-5 cells: evidence for an enhanced extrusion of calcium

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Redox modulation is involved in the regulation of the intracellular free calcium concentration ($[Ca^{2+}]_i$) in several cell types. In thyroid cells, including thyroid FRTL-5 cells, changes in $[Ca^{2+}]_i$ regulate important functions. In the present study we investigated the effects of the oxidizing compounds thimerosal and t-butyl hydroperoxide on $[Ca^{2+}]_i$ in thyroid FRTL-5 cells. Thimerosal mobilized sequestered calcium, and evoked modest store-dependent calcium entry. Both compounds potently attenuated the increase in $[Ca^{2+}]_i$ when store-operated calcium entry was evoked with thapsigargin. The entry of barium was not attenuated. Experiments performed with high extracellular pH, in sodium-free buffer and in the presence of vanadate suggested that thimerosal decreased $[Ca^{2+}]_i$ by activating a calcium extrusion mechanism, probably a plasma membrane Ca^{2+} -ATPase. All the observed effects were abrogated by the reducing agent β -

mercaptoethanol. The mechanism of action was apparently mediated via activation of protein kinase C, as thimerosal potently stimulated binding of [³H]phorbol 12,13-dibutyrate, and was without effect on store-operated calcium entry in cells treated with staurosporine or in cells with down-regulated protein kinase C. Thimerosal did not depolarize the membrane potential, as evaluated using patch-clamp in the whole-cell mode. In immunoprecipitates obtained with an antibody against plasma membrane Ca^{2+} -ATPase, we observed several phosphorylated bands in cells stimulated with thimerosal. In conclusion, we have shown that thimerosal attenuates an increase in $[Ca^{2+}]_i$, probably by activating a plasma membrane Ca^{2+} -ATPase.

Key words: Ca^{2+} -ATPase, calcium pumping, capacitative calcium entry, non-excitable cells.

INTRODUCTION

In electrically non-excitable cells, changes in the intracellular free calcium concentration ($[Ca^{2+}]_i$) induced by agonist-evoked stimulation of phospholipase C and the concomitant hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) result in the release of sequestered calcium. In addition, and temporally in very close conjunction with the release of sequestered calcium, store-operated calcium entry is activated [1,2]. The magnitude of calcium entry is also related to the amount of calcium released. Thus the calcium stores exert a regulatory function on calcium entry, and the magnitude of calcium release and the concomitant calcium entry may have an important regulatory function on cellular events. Furthermore, patch-clamp experiments have shown directly that the release of sequestered calcium can activate an inward calcium current, I_{CRAC} [3].

In addition to the well defined messenger molecules such as IP_3 that modulate calcium release, other compounds may also modify the release process. Of these compounds, several oxidizing or alkylating compounds have been investigated extensively. Thimerosal and t-butyl hydroperoxide (tBHP) in particular have been shown to be potent modulators of the release of sequestered calcium, apparently by sensitizing the IP_3 receptor to resting levels of IP_3 [4–8], and by sensitizing the ryanodine receptor [9–11]. Thimerosal also activates I_{CRAC} [12]. In addition to the aforementioned effects, these compounds may inhibit the endoplasmic Ca^{2+} -ATPase [6,13–16].

In thyroid cells, as in several other cell types, changes in $[Ca^{2+}]_i$ regulate a multitude of cellular functions. Studies have shown

that changes in $[Ca^{2+}]_i$ evoked by ATP, carbachol, noradrenaline or thyroid-stimulating hormone (TSH) may regulate the efflux of I^- [17–21], cellular proliferation [22–24], the expression of receptors for TSH [25] and the generation of H_2O_2 [26–30]. Thus, since changes in $[Ca^{2+}]_i$ are important in regulating thyroid function, and since oxidizing compounds modulate cellular calcium concentrations, we wanted to investigate the effects of thimerosal on calcium fluxes in thyroid FRTL-5 cells. Our results show that thimerosal potently activates the extrusion of cytosolic calcium, probably by activating a plasma membrane Ca^{2+} -ATPase. Furthermore, this effect seems to be mediated via a mechanism dependent on protein kinase C (PKC) activation.

MATERIALS AND METHODS

Materials

Culture medium, serum and hormones for cell culture were purchased from Gibco (Grand Island, NY, U.S.A.), Biological Industries (Beth Haemek, Israel) and Sigma (St. Louis, MO, U.S.A.). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, U.S.A.). Thimerosal, tBHP, DL-dithiothreitol, staurosporine and PMA were purchased from Sigma, hydrogen peroxide was from Riedel-de Haen AG (Seelze, Germany) and β -mercaptoethanol was from Fluka Chemie AG (Buchs, Germany). SKF96365 was generously donated by Dr. J. Merritt (SmithKline Beecham, Welwyn Garden City, U.K.). Monoclonal anti- Ca^{2+} -ATPase antibody (5F10) was from Sigma, and was also generously donated by Dr. John T. Penniston (Mayo Clinic, Rochester, MN, U.S.A.). Fura 2 acetoxymethyl ester and bisoxonol were purchased from Molecular Probes Inc. (Eugene,

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; tBHP, t-butyl hydroperoxide; IP_3 , inositol 1,4,5-trisphosphate; PKC, protein kinase C; I_{CRAC} , calcium-release-activated current; TSH, thyroid-stimulating hormone; HBSS, Hepes-buffered saline solution.

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OR, U.S.A.). Thapsigargin was from LC Services Corp (Woburn, MA, U.S.A.). The enhanced chemiluminescence detection kit, [^3H]phorbol 12,13-dibutyrate, [^{32}P]ATP and [^{32}P]P_i were from Amersham (Amersham, Bucks., U.K.). Phosphocellulose paper P81 was from Whatman (UK). Microtitration plates used in the [^3H]phorbol 12,13-dibutyrate binding assay, and the liquid scintillation cocktails Optiphase SuperMix and HiSafe 3, were from Wallac (Turku, Finland). All other chemicals used were of reagent grade. Bovine TSH was a gift from the NHPP (NIH, Bethesda, MD, U.S.A.).

Cell culture

Rat thyroid FRTL-5 cells were initially 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 [31] (insulin, 10 $\mu\text{g}/\text{ml}$; transferrin, 5 $\mu\text{g}/\text{ml}$; 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 5% $\text{CO}_2/95\%$ air at 37 °C. Before an experiment, cells from one donor culture dish were harvested with a 0.1% (w/v) 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 [^3H]phorbol 12,13-dibutyrate binding assay, the cells were grown on microtitration plates and were used when cells had reached confluency (3–4 days after plating).

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

The medium was aspirated, and the cells were harvested with Hepes-buffered saline solution (HBSS: NaCl, 118 mM; KCl, 4.6 mM; glucose, 10 mM; CaCl_2 , 1.0 mM; Hepes, 20 mM, 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 for 30 min at 37 °C. Following the loading period, the cells were washed twice with HBSS, 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 1% 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 the pH above 8.3, to obtain minimal fluorescence. $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. [32], using a computer program designed for the fluorimeter, using a K_d value of 224 nM for fura 2.

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

The assay was performed as described by Trilivas and Brown [33], 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. Then vehicle or thimerosal was added for 60 s, and the cells were washed three times with ice-cold HBSS. [^3H]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 [^3H]phorbol 12,13-dibutyrate per well.

PKC extraction and assay

Rat brain PKC was extracted and assayed as previously described [34,35], with some modifications. Briefly, a rat brain was homogenized in ice-cold Ca^{2+} -free 10 mM Hepes buffer (pH 7.5) with 100 μM leupeptin and 1 mM PMSF. Crude particles were removed by centrifuging the suspension at 200 g (10 min at 4 °C). The supernatant was further centrifuged (100 000 g; 60 min at 4 °C) without detergents. The resulting supernatant was partially purified by DEAE-Sephadex (Pharmacia) column chromatography. The protein content in the fractions was measured as described by Bradford [36]. The fraction showing the highest Ca^{2+} - and phospholipid-dependent kinase activity was stored in 50% (v/v) glycerol at -20 °C.

Before the assay, thimerosal and the PKC-containing protein extract were mixed and incubated for 15 min. The assay was started by adding the substrate to the mixture. The final reaction mixture (MgCl_2 , 7 mM; EDTA, 0.5 mM; EGTA, 0.25 mM; substrate peptide FKKFFKL-NH₂, 0.08 mM; Hepes, 10 mM, 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 $\mu\text{g}/\text{ml}$) and 1,2-*sn*-dioctanoylglycerol (8 $\mu\text{g}/\text{ml}$). 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 filter was dried and the trapped radioactivity was counted. PKC activity was calculated by subtracting non-specific activity from total activity. PKC activity was calculated as nmol of ATP transferred to the substrate/min per mg of protein extract. The kinase activities obtained were plotted semi-logarithmically, and EC_{50} values were calculated (non-linear regression; GraphPad Prism 2.0b for Macintosh).

Measurement of Ca^{2+} -ATPase phosphorylation

The cells were grown on 100-mm dishes in normal growth medium containing serum and six hormones, as described above. The method was modified from that of Kuo et al. [37]. Confluent cells were washed three times in phosphate-free minimal essential medium and incubated in the same medium containing [^{32}P]P_i (50 $\mu\text{Ci}/\text{ml}$) for 4 h in a water-saturated atmosphere of 5% $\text{CO}_2/95\%$ air at 37 °C. The test compounds were added for 15 min. The incubation was stopped by removing the medium and washing the cells with cold PBS (NaCl, 150 mM; sodium phosphate, 10 mM, pH 7.4). The cells were scraped and homogenized in 0.8 ml of lysis buffer (NaCl, 150 mM; PMSF, 1 mM; EDTA, 5 mM; EGTA, 5 mM; sodium vanadate, 100 mM; NaF, 50 mM; dithiothreitol, 2 mM; Tris/HCl, 10 mM, pH 7.4, containing 1% Nonidet P40, 1% BSA and 5 mg/ml leupeptin). The cells were extracted in lysis buffer for 1 h. Cells debris was removed by centrifugation (20 000 g for 30 min at 4 °C) and the lysates were cleared by incubation with 5 $\mu\text{g}/\text{ml}$ rabbit anti-mouse immunoglobulin/5% (w/v) Protein A-Sepharose/3 ml normal ascites fluid for 40 min at 4 °C. The cleared lysates were then incubated for 12–16 h at 4 °C with 3 μl of the 5F10 monoclonal antibody with gentle shaking. Subsequently, 5 $\mu\text{g}/\text{ml}$ rabbit anti-mouse immunoglobulin was added and the incubation was continued for 30 min. Immunocomplexes were adsorbed to 5% (w/v) Protein A-Sepharose at 4 °C for 1 h with gentle shaking. The mixture was centrifuged (200 g for 1 min at 4 °C) and washed five times with lysis buffer (as above, but without BSA). Then 50 μl of Lamli sample buffer was added to the pellet and the complex was dissociated by treatment at 92 °C for 5 min. The samples were then centrifuged (200 g for 1 min at 4 °C) and the supernatants were stored at -20 °C. The proteins

were separated on an SDS/10%-polyacrylamide gel, whereafter the gel was dried and autoradiographed.

Electrophysiological measurements of membrane potential

Electrophysiological recordings were performed using an EPC-9 amplifier and 'Pulse/PulseFit' software (HEKA Elektronik, Lambrecht/Pfaltz, Germany). The pipettes had a resistance of 5–6 M Ω when filled with intracellular solution (KCl, 140 mM; CaCl₂, 1 mM; EGTA, 11 mM; Hepes, 10 mM, pH 7.3; calculated free Ca²⁺ concentration less than 100 nM). Cells grown on small round coverslips were transferred to the experimental chamber (volume ~ 500 μ l) immediately before use and washed extensively with extracellular medium (Hepes-buffered Dulbecco's modified saline solution: NaCl, 136 mM; KCl, 2.6 mM; Na₂HPO₄, 1.46 mM; CaCl₂, 0.9 mM; MgCl₂, 0.49 mM; Hepes, 20 mM, pH 7.4). After formation of the whole-clamp configuration [38], the amplifier was switched to 'slow' current-clamp mode and the recordings were initiated about 2 min after stabilization of a cell membrane potential. Thimerosal was applied to the bath using a peristaltic pump (perfusion rate 3 ml/min). *Post hoc* statistical analysis and graphic presentation was performed using Microsoft Excel Software.

Statistics

Results are expressed as mean $\pm$ S.E.M. Statistical analysis was carried out using Student's *t* test for paired observations. When three or more means were compared, analysis of variance was used. The [³H]phorbol 12,13-dibutyrate binding data were analysed for homogeneity of group variances by 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 Mann–Whitney *U* test and Dunn–Sidak correction was performed. The calcium traces shown are representative of

at least four separate experiments from at least two batches of cells. Usually the traces shown in a Figure are from the same batch of cells.

RESULTS

Modulatory effects of thimerosal on [Ca²⁺]_i in FRTL-5 cells

In several cell types, thimerosal enhances the release of sequestered calcium by sensitizing the IP₃ receptor to endogenous levels of IP₃ [4–8], or by inhibiting the endoplasmic reticulum Ca²⁺-ATPase [6,13–16]. Challenging FRTL-5 cells with thimerosal evoked an increase in [Ca²⁺]_i (Figure 1), which was concentration-dependent (results not shown). Stimulation of FRTL-5 cells with the Ca²⁺-ATPase inhibitor thapsigargin resulted in the release of sequestered calcium, apparently from an IP₃-sensitive calcium store, and in store-operated calcium entry. Application of thapsigargin to cells treated with thimerosal resulted in a rapid

Figure 2 Thimerosal evokes store-operated calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. The cells were stimulated with 100 μ M thimerosal (trace a) or vehicle (trace b) in a calcium-free buffer containing 100 μ M EGTA, and then calcium (final concentration 1 mM) was added as indicated.

Figure 1 Effect of thimerosal on [Ca²⁺]_i 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 stimulated first with 100 μ M thimerosal (Thi) and then with 2 μ M thapsigargin (Tg). (B) The cells were stimulated with 2 μ M thapsigargin only. (C) The cells were treated with 100 μ M thimerosal, then 1 mM β -mercaptoethanol (β -m) was added, and finally the cells were stimulated with 2 μ M thapsigargin. (D) Control cells (left bars in each group of three), cells treated with 100 μ M thimerosal (middle bars) and cells treated first with thimerosal and then with 1 mM β -mercaptoethanol (right bars) were stimulated with 2 μ M thapsigargin, and the peak and plateau phases of the increases in [Ca²⁺]_i were determined. Each bar gives the mean $\pm$ S.E.M. of 4–6 determinations.

Figure 3 Thimerosal attenuates store-operated calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (A) The cells were stimulated with 2 μ M thapsigargin (Tg) in a calcium-free buffer containing 100 μ M EGTA, and then vehicle (trace a) or 100 μ M thimerosal (Thi; trace b) was added, and finally calcium (final concentration 1 mM) was added. Trace c is from control cells after addition of calcium only. (B) Thimerosal inhibited in a concentration-dependent manner the transient increase in $[Ca^{2+}]_i$ ($\square$) and the new plateau level of $[Ca^{2+}]_i$ ($\blacksquare$) when calcium (final concentration 1 mM) was added to cells stimulated with thapsigargin. Results are means $\pm$ S.E.M. of 4–7 determinations. (C) The cells were stimulated with 2 μ M thapsigargin in a calcium-free buffer containing 100 μ M EGTA and 1 mM β -mercaptoethanol. Then vehicle (trace a) or 100 μ M thimerosal (trace b) was added, and finally calcium (final concentration 1 mM) was added.

increase in $[Ca^{2+}]_i$ (Figure 1). In cells treated with concentrations of thimerosal lower than 100 μ M, the rapid thapsigargin-evoked increase in $[Ca^{2+}]_i$ was not different from that seen in control cells (results not shown). In cells treated with 100 μ M thimerosal, the rapid thapsigargin-evoked increase in $[Ca^{2+}]_i$ was significantly ($P < 0.05$) attenuated (Figure 1). This result suggested that thimerosal mobilized calcium from the same intracellular compartment as did thapsigargin. However, incubating the cells with 100 μ M thimerosal did not totally empty the thapsigargin-sensitive store, as thapsigargin in these cells transiently increased $[Ca^{2+}]_i$ by 103 ± 27 nM. The thapsigargin-evoked plateau level of $[Ca^{2+}]_i$ was decreased (Figure 1). Addition of the reducing agent β -mercaptoethanol (final concentration 1 mM) to cells treated with 100 μ M thimerosal rapidly abrogated the increase in $[Ca^{2+}]_i$ evoked by thimerosal, and fully restored the rapid increase in $[Ca^{2+}]_i$ evoked by thapsigargin (Figure 1). The plateau phase was also significantly restored, but it did not reach the level obtained in control cells (Figure 1). Probably the short duration of the incubation with β -mercaptoethanol was insufficient to totally

Figure 4 Modulation of store-operated calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (A) The cells were stimulated with 2 μ M thapsigargin (Tg) in a calcium-free buffer containing 100 μ M EGTA, and then vehicle (trace a) or 100 μ M thimerosal (Thi; trace b) was added, and finally barium (final concentration 1 mM) was added. For trace c, SKF96365 (final concentration 30 μ M) was added to the cell suspension prior to the addition of thapsigargin. The change in intracellular Ba^{2+} concentration is shown, in arbitrary units. (B) The cells were stimulated with 2 μ M thapsigargin in a calcium-free buffer containing 100 μ M EGTA in which the pH was adjusted to 8.5 and then vehicle (trace a) or 100 μ M thimerosal (trace b) was added, and finally calcium (final concentration 1 mM) was added. For trace c, SKF96365 (final concentration 30 μ M) was added to the cell suspension prior to addition of thapsigargin. For trace d, only calcium was added to the cell suspension. The change in $[Ca^{2+}]_i$ is shown, in arbitrary units. (C) The cells were pretreated with 2 mM sodium orthovanadate for 15 min. They were then stimulated with 2 μ M thapsigargin in a calcium-free buffer containing 100 μ M EGTA, and then vehicle (trace a) or 100 μ M thimerosal (trace b) was added, and finally calcium (final concentration 1 mM) was added.

restore the thapsigargin-evoked plateau level of $[Ca^{2+}]_i$. Similar results were obtained when dithiothreitol was used as a reducing agent (results not shown).

The above results suggest that thimerosal apparently mobilizes sequestered calcium. As this would result in store-operated calcium entry, we tested whether thimerosal itself could evoke calcium entry. Stimulation of FRTL-5 cells with 100 μ M thimerosal in a calcium-free buffer increased $[Ca^{2+}]_i$ by 75 ± 11 nM (Figure 2). Re-addition of calcium (final concentration 1 mM) to these cells resulted in the enhanced entry of calcium (Figure 2). This is in agreement with earlier results showing that thimerosal may indeed activate calcium entry [12]. Thimerosal apparently released calcium from the same store as did thapsigargin, as the thapsigargin-evoked increase in $[Ca^{2+}]_i$ was only 67 ± 13 nM in cells pretreated with 100 μ M thimerosal for 2 min, compared

Figure 5 Thimerosal inhibits calcium entry in a Na^+ -free buffer

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. The cells were stimulated with $2 \mu\text{M}$ thapsigargin (Tg) in a calcium- and sodium-free buffer containing $100 \mu\text{M}$ EGTA, and then vehicle (trace a) or $100 \mu\text{M}$ thimerosal (Thi; trace b) was added, and finally calcium (final concentration 1 mM) was added.

with $142 \pm 12 \text{ nM}$ in control cells ($P < 0.05$). These experiments were also performed in a calcium-free buffer.

The lack of a plateau in $[\text{Ca}^{2+}]_i$ after stimulation of thimerosal-treated cells with thapsigargin suggested either that thimerosal already had evoked a maximal entry of calcium or that thimerosal somehow interfered with the thapsigargin-evoked calcium entry. To investigate this further, cells were first stimulated with thapsigargin in a calcium-free buffer, then thimerosal was added, and finally calcium was added. In these experiments, thimerosal potently abrogated calcium entry in a concentration-dependent manner (Figure 3). If thimerosal was applied first, and the cells were then stimulated with thapsigargin, a similar attenuation of calcium entry was observed (results not shown). In the next series

of experiments, 1 mM β -mercaptoethanol was included. In these experiments, the increase in $[\text{Ca}^{2+}]_i$ after re-addition of calcium to thapsigargin-stimulated cells was similar in thimerosal-treated cells to that in control cells (Figure 3).

The observation that thimerosal apparently attenuated calcium entry was unexpected. To evaluate whether thimerosal indeed inhibits calcium entry, the above experiments were repeated, but instead of calcium, barium was added to the cells. As barium is a very poor substrate for the Ca^{2+} -ATPase [39], it cannot be extruded from the cells or sequestered in them. In Figure 4, it is clearly seen that no difference in the entry of barium could be detected between control cells and cells treated with $100 \mu\text{M}$ thimerosal. For comparison, the calcium-entry inhibitor SKF96365 [40] was tested. This compound almost totally inhibited barium entry (Figure 4). In the next series of experiments, the extracellular pH was elevated to approx. 8.5. Several investigations have suggested that H^+ ions are counterions for calcium [41,42]. In an alkaline buffer the Ca^{2+} -ATPase should thus work less efficiently, resulting in an enhanced increase in $[\text{Ca}^{2+}]_i$ after stimulation [43–46]. Again it was almost impossible to obtain a difference between control cells and cells treated with $100 \mu\text{M}$ thimerosal (Figure 4). SKF96365 again inhibited the increase in $[\text{Ca}^{2+}]_i$ in these experiments (Figure 4). With cells incubated for 30 min with 1 mM orthovanadate, an inhibitor of Ca^{2+} -ATPase [47], no difference was observed between control cells and cells treated with $100 \mu\text{M}$ thimerosal. However, the overall increase in $[\text{Ca}^{2+}]_i$ was also attenuated in control cells treated with orthovanadate, making interpretation difficult. In experiments performed in a Na^+ -free buffer, thimerosal again decreased thapsigargin-evoked calcium entry, excluding an effect mediated via Na^+ - Ca^{2+} exchange (Figure 5). In conclusion, the above results strongly suggest that thimerosal attenuates calcium entry by stimulating a Ca^{2+} -ATPase in FRTL-5 cells.

Figure 6 tBHP inhibits calcium entry

The cells were harvested and loaded with fura 2 as described in the Materials and methods section. (A) Vehicle (trace a) or $300 \mu\text{M}$ tBHP (trace b) was added to the cells, and then the cells were stimulated with $2 \mu\text{M}$ thapsigargin (Tg) in a calcium-containing buffer. (B) tBHP inhibited in a concentration-dependent manner the transient increase in $[\text{Ca}^{2+}]_i$ ($\square$) and the new plateau level of $[\text{Ca}^{2+}]_i$ ($\blacksquare$) in cells stimulated with thapsigargin. Results are means $\pm$ S.E.M. of 4–7 determinations. (C) The cells were stimulated with $2 \mu\text{M}$ thapsigargin in a calcium-free buffer containing $100 \mu\text{M}$ EGTA, and then vehicle (trace a) or $300 \mu\text{M}$ tBHP (trace b) was added, and finally calcium (final concentration 1 mM) was added. (D) tBHP inhibited in a concentration-dependent manner the transient increase in $[\text{Ca}^{2+}]_i$ ($\square$) and the new plateau level of $[\text{Ca}^{2+}]_i$ ($\blacksquare$) when calcium (final concentration 1 mM) was added to cells stimulated with thapsigargin. Results are means $\pm$ S.E.M. of 4–7 determinations.

Figure 7 Inhibition of PKC abrogates the effect of thimerosal 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 μ M PMA for 24 h. Then the cells were stimulated with 2 μ M thapsigargin (Tg) in a calcium-free buffer containing 100 μ M EGTA, next vehicle (trace a) or 100 μ M thimerosal (Thi; trace b) was added, and finally calcium (final concentration 1 mM) was added. (B) The cells were pretreated with 200 nM staurosporine for 10 min. The cells were stimulated with 2 μ M thapsigargin in a calcium-free buffer containing 100 μ M EGTA, next vehicle (trace a) or 100 μ M thimerosal (trace b) was added, and finally calcium (final concentration 1 mM) was added.

tBHP attenuates calcium entry

As the effect of thimerosal may not be very specific, two other oxidizing compounds, i.e. tBHP and 2,2'-dithiopyridine, were tested. These compounds have been shown to exert similar effects on $[Ca^{2+}]_i$ as those reported for thimerosal [8,48]. A short pre-incubation with tBHP showed that this compound had only a marginal effect on basal $[Ca^{2+}]_i$ (Figure 6). However, tBHP attenuated the thapsigargin-evoked increase in $[Ca^{2+}]_i$, both in a calcium buffer and when calcium was re-added to cells stimulated with thapsigargin in a calcium-free buffer (Figure 6). tBHP was ineffective in attenuating the entry of Ba^{2+} compared with control cells (results not shown). 2,2'-Dithiopyridine could not be tested due to extensive interference with the fura 2 fluorescence (results not shown).

Mechanism of action of thimerosal

The above results strongly suggest that thimerosal may activate a plasma membrane Ca^{2+} -ATPase, thus resulting in calcium extrusion and a decrease in $[Ca^{2+}]_i$. The activation of Ca^{2+} -ATPase has in some cell systems been evoked by PKC-mediated phosphorylation [37,49–51]. Thus we investigated whether the effect of thimerosal is mediated via a PKC-dependent mechanism. In cells that had been pretreated for 24 h with 2 μ M PMA, which results in down-regulation of the α , δ and ϵ isoforms of PKC in FRTL-5 cells [52], the inhibitory effect of 100 μ M thimerosal was totally abolished (Figure 7). In cells treated with the PKC inhibitor staurosporine (200 nM for 10 min), the effect of 100 μ M thimerosal on the thapsigargin-evoked increase in $[Ca^{2+}]_i$ was again totally abolished (Figure 7). The results thus suggest that thimerosal affects $[Ca^{2+}]_i$ via a mechanism dependent on the activation of PKC.

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

To test the effect of thimerosal on PKC activity, we measured the binding of [3 H]phorbol 12,13-dibutyrate to PKC [33]. In these experiments, thimerosal increased binding of [3 H]phorbol 12,13-dibutyrate in a concentration-dependent manner (Figure 8). However, when the effect of thimerosal on the activation of PKC was tested using a PKC preparation from rat brain, thimerosal potently inhibited PKC activity. The basal PKC activity was 144 nmol/min per mg. Addition of thimerosal (0.7–10 μ M) reduced the specific PKC activity in a concentration-dependent

Figure 8 Effect of thimerosal on [3 H]phorbol 12,13-dibutyrate binding

The cells were grown as described in the Materials and methods section. The dose-dependent effect of thimerosal on the binding of [3 H]phorbol 12,13-dibutyrate is shown. The cells were incubated with 10 nM [3 H]phorbol 12,13-dibutyrate and thimerosal or vehicle for 60 s. Results are means $\pm$ S.E.M. of 9–10 determinations using two different batches of cells (* P < 0.05 compared with control cells in the absence of thimerosal).

manner. The EC_{50} for thimerosal was 3.8 μ M (two independent assays with two parallel determinations).

Lack of an effect of thimerosal on membrane potential

In a previous study, we showed that activation of PKC depolarized FRTL-5 cells, and decreased calcium entry due to a decreased electrochemical gradient for calcium [53]. We next investigated the effect of thimerosal on the membrane potential using patch-clamp in the whole-cell mode [38]. Current-clamp experiments showed that the mean resting membrane potential was 39.5 ± 3.7 mV. Thimerosal (final concentration 100 μ M) was without effect (resting potential, 38.1 ± 5.4 mV; potential after addition of thimerosal, 40.2 ± 4.4 mV).

Phosphorylation of Ca^{2+} -ATPase in response to thimerosal

The above results strongly suggest that thimerosal may activate calcium extrusion via activation of Ca^{2+} -ATPase. We thus

Figure 9 Thimerosal phosphorylates Ca^{2+} -ATPase

The cells were grown as described in the Materials and methods section. The cells were then stimulated with vehicle (lane 1), 100 μM H_2O_2 (lane 2), 100 μM thimerosal plus 1 mM β -mercaptoethanol (lane 3) or 100 μM thimerosal (lane 4). Positions of molecular mass markers (kDa) are shown on the left.

stimulated the cells with thimerosal, and were able to immunoprecipitate several phosphorylated proteins with a Ca^{2+} -ATPase-specific antibody (Figure 9). The presence of β -mercaptoethanol abolished the effect of thimerosal (Figure 9).

DISCUSSION

The present results suggest a novel effect of oxidizing compounds in cells, i.e. the enhanced extrusion of cytosolic calcium. This mechanism is probably the result of an increase in the activity of the plasma membrane Ca^{2+} -ATPase. Our conclusion is based on the following observations. Thimerosal attenuated store-operated calcium entry in the cells, but not the store-operated entry of barium. Barium is not a substrate for Ca^{2+} -ATPase [39], and cannot easily be extruded from the cells or sequestered. However, barium entry was inhibited by SKF96356, an inhibitor of store-operated calcium entry [40]. The above results thus exclude a direct inhibitory effect of thimerosal on the calcium entry pathway. Interestingly, thimerosal potently inhibits voltage-operated calcium channels in pituitary cells [54]. Furthermore, if the extracellular pH was increased to 8.5, no effect was observed with thimerosal, although SKF96356 again was effective. Although alkaline pH amplifies a leak entry of calcium [46], the very low concentration of H^+ ions probably makes the Ca^{2+} -ATPase work less efficiently due to the lack of a sufficient amount of H^+ ions to function as counter-ions for the extrusion of calcium [41,42]. Pretreatment of the cells with vanadate, an inhibitor of Ca^{2+} -ATPase [47], abrogated the effect of thimerosal. However, the specificity of vanadate is questionable, and these results should be considered with caution.

The results in the present study suggest that the oxidizing compounds abrogated thapsigargin-evoked calcium entry, apparently by activating the plasma membrane Ca^{2+} -ATPase, resulting in enhanced calcium extrusion and thus a decrease in $[\text{Ca}^{2+}]_i$. In many cell types PKC is a potent activator of Ca^{2+} -ATPase [37,49–51]. Our results strongly suggest that the effects of thimerosal may be mediated via activation of PKC, as they were inhibited by staurosporine and in cells with down-regulated PKC. It has been shown that prolonged treatment of FRTL-5 cells with PMA down-regulates the α , δ and the ϵ isoforms of PKC [52]. Furthermore, thimerosal was a potent activator of $[\text{H}]\text{phorbol}$ 12,13-dibutyrate binding. Such increased binding is not a direct indication of PKC activity, but it correlates well with the activity *in situ*. Although we cannot exclude the possibility that staurosporine treatment or down-regulation of PKC with PMA modulated calcium extrusion by some other mechanism, the present results strongly suggest a role for an effect mediated via activation of PKC. Interestingly, when a preparation of PKC

from rat brain was used, a decrease in PKC activity was obtained with thimerosal. Similar results have been obtained with the oxidizing agent H_2O_2 [55]. These results suggest that probably only a small fraction of thimerosal enters the cells, and activates PKC by an indirect mechanism. At present we do not know what this mechanism is. Alternatively, thimerosal may interact with the plasma membrane, and an oxidizing event may trigger a signalling cascade resulting in the activation of PKC.

In immunoprecipitates from cells labelled with $[\text{P}]\text{P}_i$, we obtained much stronger labelling of proteins after stimulation with thimerosal, and this effect was abolished by β -mercaptoethanol. However, we were not able to obtain a single phosphorylated band in our experiments, suggesting that the Ca^{2+} -ATPase was probably degraded during preparation. Interestingly, in alveolar macrophages, oxidizing compounds inactivate the plasma membrane Ca^{2+} -ATPase [56]. In addition to these effects, thimerosal has been reported to inhibit Ca^{2+} -ATPase in the endoplasmic reticulum [6,13–16].

Activation of PKC decreases store-operated calcium entry in several cell types [57,58], including FRTL-5 cells [53]. Our previous results also showed that activation of PKC caused depolarization of the membrane potential [53]. This results in a lowered electrochemical gradient for calcium, leading to a decreased influx of calcium. Interestingly, thimerosal did not depolarize the membrane potential, although it potently stimulated $[\text{H}]\text{phorbol}$ 12,13-dibutyrate binding. However, in HEL cells and in Jurkat T-cells, activation of the PKC β isoenzyme specifically abrogates calcium entry [59,60]. At present we do not know which isoenzymes of PKC are activated by thimerosal in our cells. It is possible that a similar mechanism to that described in HEL cells and in Jurkat T-cells also exists in FRTL-5 cells.

In conclusion, the results obtained in the present study define a new mechanism of action of oxidizing compounds in FRTL-5 cells, i.e. activation of calcium extrusion. This effect is probably mediated via a PKC-dependent mechanism.

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