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EFFECT OF THIMEROSAL AND OTHER SULFHYDRYL REAGENTS ON CALCIUM PERMEABILITY IN THYMUS LYMPHOCYTES

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Abstract—We have studied the effects of thimerosal, a mercurial compound extensively used as a preservative, as well as other sulfhydryl reagents (e.g. *p*-hydroxymercurybenzoate, hydrogen peroxide, bromophenacyl bromide, and mercuric chloride) on Ca^{2+} homeostasis and the redox status of sulfhydryl groups in thymus lymphocytes. They all induced an increase in $[Ca^{2+}]_i$ which was blocked with dithiothreitol, suggesting that they act via the oxidation or blockade of sulfhydryl groups. $[Ca^{2+}]_i$ increase could be directly related to the effect of the different reagents on cellular protein sulfhydryl content. Experiments with ethidium bromide indicate that the observed rise in $[Ca^{2+}]_i$ was not due to a non-specific increase in membrane permeability. Thimerosal differs from the other agents studied in its oxidative properties, which is probably linked to the production of a potent reductor molecule, thiosalicylic acid, which may modulate its oxidative capacity.

Key words: Ca^{2+} homeostasis; thymus lymphocytes; thimerosal; sulfhydryl reagents

Thimerosal is used as a preservative in many pharmaceutical solutions and has also been reported to elicit the production of specific antibodies and cell-mediated immunity [1, 2]. These effects are related to its oxidative capacity [2]. The redox status of a cell is an important factor in the maintenance of general cellular homeostasis, in particular calcium homeostasis [3–7]. Essential sulfhydryl groups are present in a number of membrane-bound proteins related to Ca^{2+} permeability, and it is known that sulfhydryl reagents may alter the internal Ca^{2+} concentration [8–10]. We have previously described that thimerosal induces an increase in $[Ca^{2+}]_i$ in rat thymus lymphocytes [11], as well as in other cell preparations [12–19].

The effect of thimerosal on $[Ca^{2+}]_i$ homeostasis is complex, varying with dose and cell type [15–20]. In some cases, it has been proposed that thimerosal induces an increase in $[Ca^{2+}]_i$ by sensitizing the $InsP_3$ receptor, a phenomenon also observed with oxidized glutathione and *t*-butyl hydroperoxide at higher concentrations [18, 21]. In other preparations, thimerosal seems to open a pathway for Ca^{2+} entry from the extracellular side [11, 19].

The present study analyses the effects of thimerosal and other sulfhydryl reagents, including BPB, H_2O_2, $HgCl_2$ and pHMB,† on $[Ca^{2+}]_i$ in thymus lymphocytes. Although the compounds used differed in their mechanisms of action, their effects in all cases were abolished with DTT. We provide evidence that there is a relationship between the SH-blocking capacity and the potency of these agents in increasing $[Ca^{2+}]_i$. These effects are not a general consequence of cell damage since cell viability as evidenced by LDH release and ethidium bromide uptake was not affected by brief exposure to thiol reagents.

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† Abbreviations: AA, arachidonic acid; BPB, bromophenacyl bromide; $[Ca^{2+}]_i$, internal calcium concentration; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; p-HMB, *p*-hydroxymercuribenzoate; LDH, lactate dehydrogenase; 2-ME, 2-mercaptoethanol; PHA, phytohaemagglutinin; PLA_2, phospholipase A_2; SH, sulfhydryl; TNB, 2-nitro-5-thiobenzoic acid.

MATERIALS AND METHODS

Cell isolation. Thymocytes were prepared from 6-week-old Wistar rats of either sex as previously described [11], and kept in a standard saline composed of (in mmol/L): 125 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 1.5 NaH_2PO_4, 10 glucose and 25 Hepes, pH 7.4. Standard saline was used in all experiments unless otherwise indicated.

Measurement of $[Ca^{2+}]$ *concentration.* Loading cells with Fura-2/AM and measurement of $[Ca^{2+}]_i$ were performed as previously described [11]. For measurement of $[Ca^{2+}]_i$, thymocytes were suspended in a thermostatically controlled and magnetically stirred fluorimeter cuvette (Perkin-Elmer LS5) at a concentration of $1–2 \times 10^7$ cells/mL. Since phosphate, when complexed with manganese, increases autofluorescence at the excitation wavelength of Fura-2, free phosphate was omitted from the reaction mixture in all experiments. To prevent Fura-2 leakage from the cells we used 2.5 mM probenecid. Fura-2 leakage was determined in the calibration cuvettes by the decrease in fluorescence after the addition of 100 μM $MnCl_2$. Only cell preparations with less than 1–2% fluorescence decrease (100 is the arbitrary unit for fluorescence in the presence of digitonin) were used for the experiments.

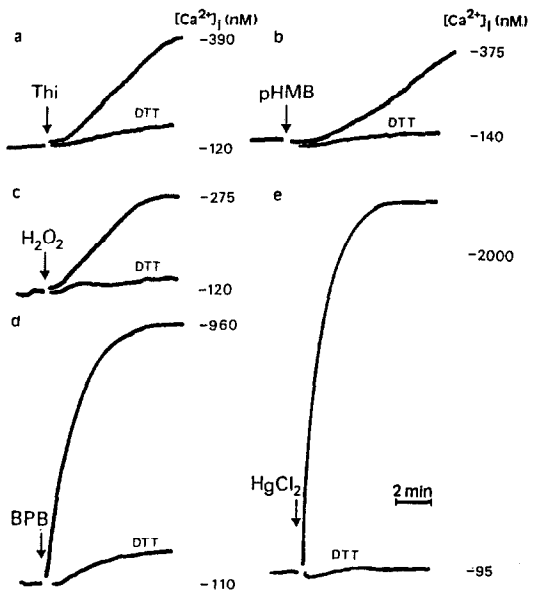

Fig. 1. DTT prevents sulfhydryl-reagent induced $[Ca^{2+}]_i$ increase. Fura-2 loaded thymocytes ($1–2 \times 10^7$/mL) were suspended in standard saline without phosphate and fluorescence was measured as explained in Materials and Methods. When indicated by arrows 20 μM thimerosal (a), 200 μM pHMB (b), 130 μM H_2O_2 (c), 50 μM BPB (d) and 10 μM $HgCl_2$ were added. Lower traces of each figure indicate the effect of the same agonists in the presence of 1 mM DTT. Basal values were very similar and are superimposed for simplicity in the figure. This experiment is representative of three similar ones performed with different thymocyte preparations.

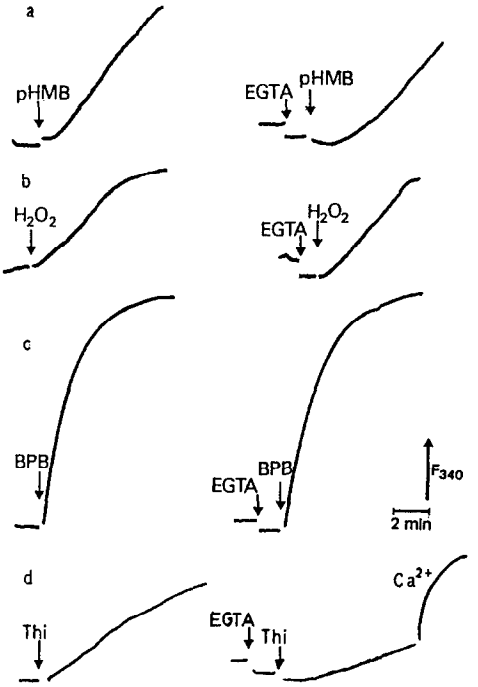

Fig. 2. Effect of a low calcium medium and EGTA on fluorescence changes in response to sulfhydryl reagents. Experimental conditions were the same as in Fig. 1. Traces at the left of the figure represent changes in fluorescence in control situation with 200 μM pHMB (a), 130 μM H_2O_2 (b), 50 μM BPB (c) and 20 μM thimerosal. Traces at the right of the figure represent changes in fluorescence with the same reagents in a nominally Ca^{2+}-free medium after addition of 400 μM EGTA. EGTA decreases basal fluorescence, probably due to the removal of Ca^{2+} bound to dye leaked out of the cells. The results are representative of three similar experiments.

Determination of sulfhydryl group oxidation or blockade. The capacity of different reagents to oxidize or block SH-groups directly was measured as described [22]. The reversible oxido-reduction of the pair (TNB/DTNB) was continuously monitored by changes in absorbance measured at 412 nm. The SH-blocking capacity of any compound is indicated by its ability to decrease the absorbance of a solution containing TNB. TNB was made by the previous reduction of 10 μM DTNB with 20 μM of 2-ME. The SH-protein content of cells was determined as described [23]. Briefly, following centrifugation ($1–2 \times 10^7$ cells/mL), cellular proteins were precipitated and then washed twice with 6.5% (w/v) trichloroacetic acid to remove acid-soluble sulfhydryls. The protein pellet was suspended in 1 mL of 500 mM Tris–HCl, 10 mM EDTA (pH 7.6) and sonicated. After adding 100 μL of 1 mM DTNB, the samples were incubated for 20 min at room temperature. The increase in absorbance, measured at 412 nm, was taken as an index of SH-content. Data are expressed as percentage of total sulfhydryl content in untreated cells.

Cell viability. Cell viability was determined either by Trypan blue exclusion or by measuring the leakage of LDH from the cells. LDH activity was determined according to standard methods. The uptake of extracellular markers such as ethidium bromide was used as an indicator of non-specific membrane permeability.

Chemicals. Fura-2/AM, AA, BPB, pHMB, and DTNB were purchased from Sigma Chemical (St Louis, MO, U.S.A.). Thimerosal was obtained from Fluka (Buchs, Switzerland). PHA was purchased from Flow Laboratories (U.S.A.). H_2O_2 was obtained from Foret (Barcelona, Spain) and the concentration in mol/L was calculated from the absorbance (abs) at 240 nm by using the following expression: M = (abs × dilution factor)/39.4 [24].

RESULTS

Sulfhydryl reagents and internal $[Ca^{2+}]$

Figure 1 shows the kinetics of the rise in $[Ca^{2+}]_i$ of lymphocytes caused by thimerosal and other sulfhydryl reagents and the inhibitory effect of DTT. The average time at which each agonist initiates Ca^{2+} increase was dependent on the compound used. With either thimerosal, pHMB or H_2O_2, $[Ca^{2+}]_i$ increased after a delay of approx. 30 sec (traces a, b and c). In contrast, with either BPB or $HgCl_2$, $[Ca^{2+}]_i$ increased without an appreciable lag and reached a maximum within 60 to 90 sec (traces d

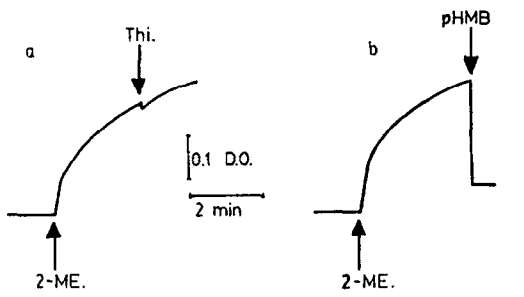

Fig. 3. SH-reactivity properties of thimerosal and pHMB on the TNB/DTNB pair in a buffer solution. The oxidative or SH-blocking capacity of thimerosal and pHMB was determined as described in Materials and Methods. After the reduction of DTNB induced by 2-ME, 50 μM of thimerosal (a) or pHMB (b) was added to the buffer.

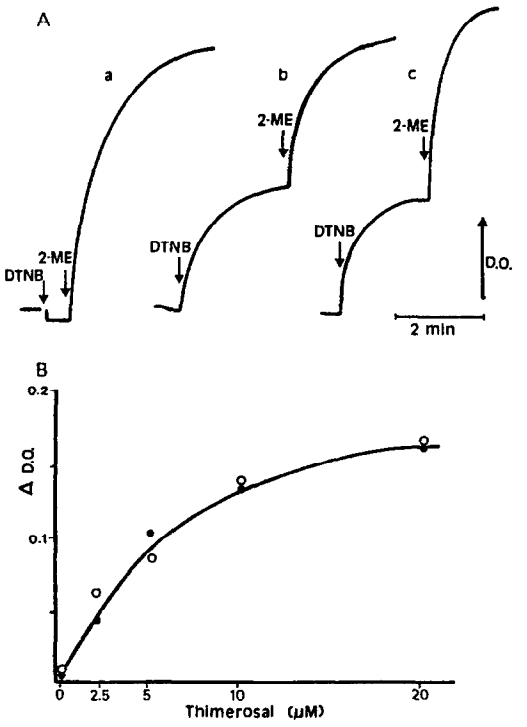

Fig. 4. Effect of thymocyte pretreatment with thimerosal on the TNB/DTNB redox pair. Thymocytes (1–2 × 10^7/mL) were incubated at 37° for 5 min with either standard buffer or 20 μM thimerosal in 1 mL total volume and absorbance at 412 nm was measured. When indicated by arrows 1 μL of 10 mM DTNB was added. After stabilization of the recording, addition of 2 μL of 10 mM 2-ME induced a complete reduction in DTNB. Traces Aa and Ab represent changes in absorbance obtained in control and treated cells, respectively. Trace Ac corresponds to changes in absorbance in the supernatant of centrifuged cells after 5 min incubation with 20 μM thimerosal. Panel B shows the concentration dependence of the effect of 5 min incubation with thimerosal on the increments in absorbance after the addition of DTNB with either the cell suspension or the supernatant (○, ●).

and e). With most agonists, steady-state was reached in approx. 5 min, although pHMB induced a slow and continuous increase in fluorescence. Maximal increases in $[Ca^{2+}]_i$ were observed with the following concentrations: 20 μM thimerosal, 200 μM pHMB, 130 μM H_2O_2, 50 μM BPB and 10 μM $HgCl_2$. Thimerosal, pHMB and H_2O_2 increased $[Ca^{2+}]_i$ approx. 2.5 to 3-fold. However, 10 to 20-fold increases were observed with BPB and $HgCl_2$. Under the same experimental conditions DTT had no appreciable effect on the rise in $[Ca^{2+}]_i$ induced by PHA or AA, two agents known to increase $[Ca^{2+}]_i$ in thymocytes [25, 26] (results not shown). Our present data indicate that a variety of sulfhydryl reagents modify Ca^{2+} homeostasis in thymus lymphocytes. It is worth noting that thiosalicylic acid, a compound derived from thimerosal, has no effect on $[Ca^{2+}]_i$ concentration (results not shown).

In order to analyse the dependence of thiol-reagent-dependent changes in $[Ca^{2+}]_i$ on external Ca^{2+} we incubated the cells in a Ca^{2+}-free medium (with 400 μM EGTA added). Figure 2 illustrates that the increase in $[Ca^{2+}]_i$ induced by pHMB (a), H_2O_2 (b) and BPB (c) was independent of external Ca^{2+}. These facts suggest that $[Ca^{2+}]_i$ may rise either due to release of Ca^{2+} from intracellular stores or inhibition of any of the calcium pumps or exchangers related to the Ca^{2+} homeostasis of the cell. Thimerosal, however, exhibited more complex behaviour, since in most cases $[Ca^{2+}]_i$ did not increase in the absence of external Ca^{2+} (Fig. 2, trace d). In 15–20% of the cellular preparations, however, thimerosal induced an increase in $[Ca^{2+}]_i$ even in the absence of external Ca^{2+} (not shown).

Interaction of the agents studied with sulfhydryl groups

To investigate further the SH-blocking capacity of thimerosal and the other agents, we analysed the oxidation or SH-blockade of TNB as described in Material and Methods. The potency of the different agents to either oxidize or block SH-groups of TNB was variable. Whereas pHMB, BPB and $HgCl_2$ (at the concentrations used to measure $[Ca^{2+}]_i$) drastically reduced absorbance at 412 nm, H_2O_2 had a slower effect on the oxidation or blockade of the SH-group of TNB. Unlike the other compounds tested, thimerosal seemed neither to oxidize TNB nor to reduce DTNB at any concentration used. For example, Fig. 3 illustrates the effects of thimerosal and pHMB, two mercurials that presumably interact with similar groups, on the TNB/DTNB pair, in a protein-free solution. Nevertheless, when the cells were incubated with 20 μM thimerosal (for 5 min), the addition of DTNB produced an increase in absorbance not observed in control cells (without thimerosal) (Fig. 4A, traces a and b). This indicates the release of a reductor agent through the interaction of thimerosal with the cells. The capacity to reduce DTNB was lost if the cells were centrifuged and then resuspended in fresh buffer (trace similar to a) but could be shown to be present in the supernatant (Fig. 4A, trace c). The effect of thimerosal was dependent on the concentration used. Figure 4B

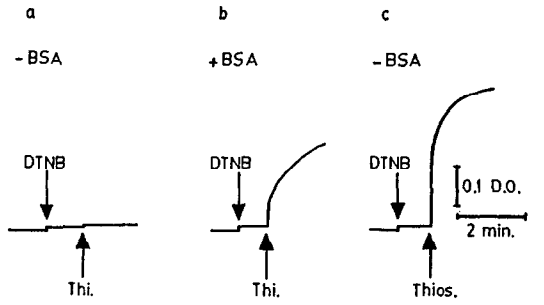

Fig. 5. Effect of thimerosal and thiosalicylic acid on the redox state of the TNB/DTNB pair. When indicated by the arrow 20 μM thimerosal was added to the solution without albumin (trace a) or with 10 mg/mL albumin (trace b). Trace c shows the direct reduction of DTNB by 20 μM thiosalicylic acid in a buffer without albumin.

Table 1. Influence of different thiol reagents on the protein sulfhydryl content of thymus lymphocytes

Oxidants	Sulfhydryl content (%) 3 min	5 min	60 min
Thimerosal (20 μM)	96 ± 1*	95 ± 1*	101 ± 5
BPB (50 μM)	73 ± 4*	74 ± 7*	72 ± 9*
H_2O_2 (130 μM)	94 ± 3*	97 ± 2	102 ± 5
$HgCl_2$ (10 μM)	29 ± 1*	31 ± 2*	47 ± 7*
pHMB (200 μM)	92 ± 2*	95 ± 1*	62 ± 6*

Thymocytes (1–2 × 10^7/mL) were preincubated with the different compounds for 3, 5 or 60 min. SH-protein content was measured as described in Materials and Methods. Results are normalized with respect to the SH-content in control cells (100). Data represent the mean ± SD of three different experiments.

* Significantly different from control ($P < 0.05$).

shows the increase in absorbance after addition of DTNB to either cells incubated with different concentrations of thimerosal (filled circles) or supernatant collected after the centrifugation of the cells (open circles). Given the molecular structure of thimerosal these results suggest that thimerosal probably interacts with cellular SH-groups producing free thiosalicylic acid which could be the reductor agent found in the supernatant. To test this hypothesis we compared the effects of thimerosal and thiosalicylic acid on the oxido-reduction state of the TNB/DTNB pair in the absence of cells. The results are illustrated in Fig. 5. Although thimerosal alone was unable to reduce DTNB (line a) this capacity was present when albumin was added to the buffer (line b). A similar response was observed when thiosalicylic acid was added in the absence of albumin (line c). Further support for the formation of thiosalicylic acid after incubation with thimerosal was obtained by testing the well-known property of this reductant, i.e. its ability to form a blue complex with $FeCl_3$ (not shown).

The disruption of Ca^{2+} homeostasis could result from the depletion of soluble and protein-bound thiols [9]. Table 1 summarizes the protein sulfhydryl content of thymocyte after exposure of the cells to the studied compounds, expressed as the percentage of sulfhydryl content in non-treated cells. Variable results are observed when the cells are incubated for 3 or 5 min with different agents. $HgCl_2$ produced the most pronounced reduction in SH content, followed by BPB. Thimerosal, pHMB and H_2O_2, however, caused only a slight decrease in the SH-group content ($P < 0.05$). With pHMB, this decrease was most evident at longer incubation times. With thimerosal and H_2O_2, however, full recovery of SH-groups was evident at 60 min incubation time. These data are in agreement with previous observations which show a relationship between depletion of SH-groups and Ca^{2+} permeability [9].

Membrane permeability

Non-specific membrane permeability induced by sulfhydryl reagents was determined by ethidium bromide uptake following the addition of different compounds. Table 2 shows that none of the agents tested induced ethidium bromide uptake at the time that $[Ca^{2+}]_i$ reached its maximum value (from 1 to 5 min). Furthermore, thimerosal, BPB and H_2O_2 did not modify membrane permeability at any time studied compared to control cells. However, a drastic increase was observed after 60 min with pHMB and after 30 min with $HgCl_2$. LDH leakage gave similar results (not shown). These data argue against membrane leakage resulting from the tested agents and thereby giving rise to elevated $[Ca^{2+}]_i$.

DISCUSSION

In this study we compared the effects of several sulfhydryl blocking reagents on Ca^{2+} homeostasis in thymus lymphocytes. Even if the main action of some of the agents tested is not related to thiol groups, the increase of $[Ca^{2+}]_i$ they all induced should involve SH-groups since it was abolished by the presence of dithiothreitol. Special attention was paid to the relative oxidative potency of thimerosal, a mercurial compound used as a preservative in many pharmaceutical applications [18, 19], especially to oxidize or block protein-associated sulfhydryl groups.

The ability of thiol reagents to modify cellular Ca^{2+} homeostasis is well known [6–8]. However, their mechanism of action may vary depending on the agent used and the cell type. In thymocytes, $[Ca^{2+}]_i$ increase could be directly related to the effect of the different reagents on cellular protein-sulfhydryl content. Thus, compounds that only slightly modified the SH-content of thymocytes, such as H_2O_2, pHMB and thimerosal, produced a moderate $[Ca^{2+}]_i$ increase. In contrast, $HgCl_2$ and BPB, which markedly decreased the protein sulfhydryl content, produced a more pronounced $[Ca^{2+}]_i$ increase.

In solutions without proteins, thimerosal did not seem to either oxidize TNB or reduce DTNB. Interestingly, when cells were first incubated with thimerosal and later exposed to DTNB, this compound became rapidly reduced. Since the capacity to reduce DTNB was found in the

Table 2. Influence of different sulfhydryl reagents on non-specific membrane permeability

Addition	Ethidium bromide uptake Incubation time (min) 0	5	30	60
None (control)	15.7 ± 0.9	14.8 ± 4.0	13.9 ± 4.7	13.8 ± 4.7
Thimerosal	13.5 ± 2.8	15.0 ± 2.0	16.4 ± 6.8	16.4 ± 5.1
H_2O_2	13.6 ± 2.9	13.5 ± 3.0	15.3 ± 5.9	15.8 ± 6.5
PBP	13.8 ± 2.8	14.2 ± 2.6	14.8 ± 3.8	18.1 ± 2.8
pHMB	15.2 ± 3.2	16.2 ± 4.0	23.8 ± 9.1	51.3 ± 9.4*
$HgCl_2$	15.9 ± 2.7	17.1 ± 3.0	40.5 ± 8.2*	59.5 ± 7.3*

Thymocytes (1–2 × 10^7/mL) were incubated for 0, 5, 30 and 60 min with thimerosal (20 μM), H_2O_2 (130 μM), BPB (50 μM), pHMB (200 μM) and $HgCl_2$ (10 μM). After the initial recording, 0.05 mg/mL ethidium bromide was added to the cell suspension and when fluorescence, measured at 366/590 (Ex/Em) nm, was stabilized the different agents were added. Data are expressed in arbitrary fluorescence units and represent the mean ± SD of three different experiments.

* Significantly different from control cells ($P < 0.001$).

supernatant and not in the cells, we assumed that thimerosal interacts with some cellular component, probably SH-cysteine groups, to produce a reductor molecule. Considering the molecular structure of thimerosal, the most likely reductor molecule would seem to be thiosalicylic acid, produced from thimerosal after interaction with the cells. This presumption was experimentally supported by the fact that thiosalicylic acid produced the same pattern of DTNB reduction as thimerosal when albumin was present in the reaction mixture. Thiosalicylic acid also imitated the effect of supernatant from cell preparations previously treated with thimerosal. The production of thiosalicylic acid was further supported by the well-known property of this reductant to form a blue colour when complexed with $FeCl_3$. To explain why thimerosal does not change the absorbance of the TNB/DTNB pair we suggest two possibilities: (a) thimerosal cannot interact directly with the TNB/DTNB pair but can interact with SH-groups of proteins, or (b) thimerosal interacts with SH-groups of TNB, but in this reaction thiosalicylic acid is produced, which can reduce disulfide bonds of DTNB. The result of these oxido-reduction reactions is that no change in absorbance is observed.

Thiosalicylic acid was unable to induce any increase in $[Ca^{2+}]_i$, suggesting that the rise in $[Ca^{2+}]_i$ induced by thimerosal is mediated by its interaction with SH-groups prior to the splitting of the molecule. However, the specific SH-group that interacts with thimerosal may differ with the cell type. In thymocytes, the rise in $[Ca^{2+}]_i$ induced by thimerosal is clearly decreased in the absence of extracellular Ca^{2+}. This dependency has also been observed in human endothelial cells [19]. In Jurkat T-cells, thimerosal induces a rise in $[Ca^{2+}]_i$ due to both release of Ca^{2+} from intracellular stores and Ca^{2+} influx [27]. In other cells, the Ca^{2+} increase induced by thimerosal is independent of external Ca^{2+} but dependent upon $InsP_3$-induced Ca^{2+} release [10, 15, 17]. In thymocytes, the contribution of internal calcium stores and the role of the $InsP_3$ receptors in the increase of $[Ca^{2+}]_i$ needs to be investigated further. Recently, a membrane-associated $InsP_3$ receptor that can induce Ca^{2+} influx has been described in lymphocytes [28].

The present work also provides evidence that in thymocytes different types of oxidants enhanced $[Ca^{2+}]_i$ by a mechanism that involved sulfhydryl groups. The rise in $[Ca^{2+}]_i$ induced by inorganic mercury and H_2O_2 was independent of extracellular Ca^{2+}. In contrast, in PC12 cells, inorganic mercury induced an increase in $[Ca^{2+}]_i$ dependent on extracellular Ca^{2+} [29]; similar results have been described with H_2O_2 in cardiac muscle [30]. The different results obtained in different preparations may be due to the different concentrations used, since it is well reported in the literature that subtle changes in the critical proteins of SH-groups may induce different and even opposite effects [20, 28, 30]. Thimerosal clearly differs from other sulfhydryl reagents in its oxidative properties, probably due to the production of the reductor thiosalicylic acid which can modulate or counterbalance the oxidative action of thimerosal. BPB, broadly used as an inhibitor of PLA_2, is shown drastically to increase $[Ca^{2+}]_i$ in thymocytes mediated by its SH-blocking power. The effect of BPB on $[Ca^{2+}]_i$ increase was dose dependent and stimulates the expression of surface antigens in lymphocytes (manuscript in preparation). The effect of the agents studied was not due to non-specific membrane leakage, since no increase in ethidium bromide uptake was observed at the time that $[Ca^{2+}]_i$ reached its maximum value. These elements should be taken into account in order to explain some of the effects of these compounds extensively utilized for biochemical and pharmaceutical purposes.

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