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Effect of the sulfhydryl reagent thimerosal on cytosolic free Ca^{2+} and membrane potential of thymocytes

Anna S. Gukovskaya, Elena S. Trepakova, Valery P. Zinchenko, Yuri N. Korystov and Vladimir V. Bezuglov

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region (Russia)

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The sulfhydryl reagent thimerosal at concentrations 5–100 μM has been found to induce a variety of changes in ion transport in rat thymocytes. In particular, $[Ca^{2+}]_i$ increases about 10-fold from the basal level. The $[Ca^{2+}]_i$ response to thimerosal displays a two-stage time course, with the main $[Ca^{2+}]_i$ rise during the second stage. Evidence has been obtained for the depletion of intracellular Ca^{2+} pools in thimerosal-treated cells, however, Ca^{2+} mobilization from intracellular stores does not contribute significantly into $[Ca^{2+}]_i$ rise. Thimerosal elicits permeability not only for Ca^{2+}, but also for Mn^{2+} and Ni^{2+}, which is Ca^{2+}-dependent. We failed to get any evidence on thimerosal-induced inhibition of the plasma membrane Ca^{2+}-ATPase. The induction of Ca^{2+} influx, rather than inhibition of Ca^{2+}-ATPase, accounts for the disturbance of $[Ca^{2+}]_i$ homeostasis in thimerosal-treated cells. Thimerosal also elicits changes in monovalent ion fluxes resulting in marked depolarization. The latter seems unrelated to the changes in $[Ca^{2+}]_i$ and is suggested to be mediated both by increased permeability for Na^+ and a decreased one for K^+. Thimerosal significantly stimulates AA release from thymocytes. Evidence has been presented that AA metabolite(s), probably, LO product(s), may mediate the changes in the transport of mono- and divalent cations elicited by the sulfhydryl reagent. Prolonged treatment of thymocytes with thimerosal resulted in cell death.

Introduction

Sulfhydryl reagents that reduce intracellular SH level are known to induce a variety of diverse physiological and pathophysiological processes in eukaryotic cells, including secretion, aggregation and cell death [1–3]. It has been suggested recently that toxic effects of sulfhydryl reagents may be mediated by perturbation of Ca^{2+} homeostasis [3,4]. Ca^{2+} is known to play a fundamental role in physiological and pathophysiological processes in lymphocytes, including proliferation and cell death [4–6]. Little is known, however, about the action of SH reagents on $[Ca^{2+}]_i$ regulation. Their ability to interfere with Ca^{2+}-ATPases is usually considered the only pathway mediating the effect of SH group inhibitors on Ca^{2+} transport [3].

In several cell types SH reagents have been found to modulate monovalent ion transport as well [7,8]. In particular, they activate K^+/Cl^- cotransport [5] and inhibit Na^+/H^+ exchange [8]. However, there is little data available on the effects of SH reagents on the electrogenic transport of monovalent ions.

Earlier we have found that thimerosal increases $[Ca^{2+}]_i$ of rat thymocytes [9,10]. Recently this observation was confirmed by Martin et al. [11] who studied also thimerosal-induced changes in pH_i and glycolytic rate. In the present study we have addressed in detail the effects of the sulfhydryl reagent on $[Ca^{2+}]_i$ and the membrane potential of rat thymocytes. The $[Ca^{2+}]_i$ response to thimerosal has been found to display a two-stage time course with the main $[Ca^{2+}]_i$ rise during the second stage. The putative inhibition of Ca^{2+}-ATPases by the SH reagent does not seem to contribute significantly to the observed $[Ca^{2+}]_i$ rise.

Thimerosal also modifies monovalent ion fluxes in thymocytes, thus producing a pronounced depolarization. Evidence is given that this depolarization is medi-

Correspondence to: A.S. Gukovskaya, Department of Gastroenterology (111-D), VA Medical Center, 3350 La Jolla Village Drive, San Diego, CA 92161, USA.

Abbreviations: thimerosal, ethylmercurithiosalicylate; di-BA-C_4(3), bis(1,3-dibutylbarbiturate)trimethine oxonol; diS-C_2-(5), 3,3′-diethylthiadicarboxyanine iodide; SBS, standard buffered saline; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; $[X^+]_o$, ion X concentration in the external medium (e.g., $[Ca^{2+}]_o$); AA, arachidonic acid; LO, lipoxygenase; NDGA, nordihydroguiaretic acid; 5-HETE, 5*S*-hydroxy-5,8,11,13-(*Z*,*Z*,*Z*,*E*)-eicosatetraenoic acid.

ated both by a decrease in the plasma membrane permeability for K^+ and an increase in the permeability for Na^+.

Thimerosal significantly stimulates AA release from thymocytes. Evidence has been presented that AA metabolite(s), probably, LO product(s), may mediate the changes in the transport of mono- and divalent cations elicited by the sulfhydryl reagent. Prolonged treatment of thymocytes with thimerosal resulted in cell death.

Materials and Methods

Cell preparation and medium composition

Thymocytes were obtained from Wistar rats (approx. 150 g) by teasing out the thymus glands through a nylon mesh into medium 199 [10], then washed twice by centrifugation and resuspended in SBS containing 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM Na_2HPO_4, 4 mM $NaHCO_3$, 6 mM glucose, 10 mM Hepes (pH 7.2).

When a modified medium was used, the cells were transferred to such a medium just before the measurements. In the low-Na^+ medium NaCl was replaced by choline chloride and $[Na^+]_o$ was about 16 mM. The Na^+-free medium was SBS free of $NaHCO_3$ and Na_2HPO_4, where NaCl was replaced by choline chloride and the pH was adjusted by KOH ($[Na^+]_o \leq 1$ mM). In the low-Cl^- medium NaCl was replaced by sodium gluconate ($[Cl^-]_o \cong 8$ mM), and in the K^+-rich medium it was substituted by KCl ($[K^+]_o \cong 146$ mM; $[Na^+]_o \cong 16$ mM). In some experiments Ca^{2+} in the medium was reduced or omitted (as stated in figure legends); a Ca^{2+}-free SBS containing either 100 μM EGTA with no added $CaCl_2$ or 1 mM EGTA plus 0.15 mM $CaCl_2$ has been used as well.

The integrity of thymocyte plasma membrane was assessed by staining the cells with 0.04% Trypan blue or 30 μM ethidium bromide (the wavelengths for excitation and recording were 365 and 600 nm, respectively). Cell viability was expressed as the percentage of thymocytes that excluded Trypan blue.

Measurement of ion transport

$[Ca^{2+}]_i$ was measured using quin2. Thymocytes ($5 \cdot 10^7$ per ml) were loaded in SBS with 5 μM quin2 acetoxymethyl ester for 40 min at 37°C, then washed and resuspended in the same medium free of the dye. The excitation and emission wavelengths were 337 and 495 nm, and the slits 2 and 10 nm, respectively. $[Ca^{2+}]$ signals were calibrated according to the standard procedure [10,12] by adding 15 μM digitonin and 0.1 mM $MnCl_2$ to obtain the fluorescence of quin2 saturated with or free of Ca^{2+}, respectively. The dissociation constant for the Ca^{2+}-quin2 complex is 115 nM at 37°C [12].

Redistribution of intracellular Ca^{2+} was monitored using the fluorescence probe chlortetracycline, as described in [10]. Cells were incubated in SBS for 40 min at 37°C with 20 μM chlortetracycline and then directly assayed in the fluorometer. The excitation and emission wavelengths were 405 and 520 nm and the slits 3 and 8 nm, respectively.

The membrane potential was measured using the fluorescence probes diS-C_2-(5) and di-BA-C_4(3) [10,13]. The wavelengths for excitation and recording were 579 and 672 nm for dis-C_2-(5) and 580 and 650 nm for di-BA-C_4(3), respectively. The slits were 3 nm for excitation and 5 nm for recording. For both dyes an increase in the fluorescence intensity indicates depolarization of the cells, hyperpolarization being accompanied by a decrease in the fluorescence. The calibration procedure for diS-C_2-(5) was performed as described in Refs. 10 and 14 by isoosmotically varying the extracellular K^+ concentration in the presence of 2 μM K^+ ionophore valinomycin. Under these conditions the membrane potential can be calculated by Nernst equation $E_m = -61.5 \log([K^+]_i/[K^+]_o)$ with the intracellular K^+ concentration estimated as 150 nM [14].

The fluorescence was measured in a special spectrofluorometer, as described in Ref. 9, at 37°C and with continuous stirring, the concentration of cells in 2 ml thermostated cuvette was about $5 \cdot 10^6$ per ml.

After the cells had been transferred into the cuvette they were allowed to adjust for 3–4 min and a steady-state basal fluorescence level was registrated prior to application of agents.

Analysis of [^{3}H]arachidonic acid release

Thymocytes were suspended in RPMI 1640 at a concentration of $2 \cdot 10^7$ per ml and incubated with 1 μCi/ml [^{3}H]arachidonic acid for 1 h at 37°C. After radiolabelling the cells were washed three times with SBS. The cells were then incubated with or without thimerosal at 37°C. The reaction was terminated at times indicated by the addition of 2 ml chloroform/4-methanol (1 : 2, v/v). Then the cells were spun (700 × g, 5 min) and the radioactivity in the supernatant was measured by scintillation counter SL-4000 (France).

Materials

EGTA, quin2 acetoxymethylester, NDGA, indomethacin were obtained from Calbiochem; thimerosal, Hepes from Fluka; furosemide, SITS, AA from Sigma; di-BA-C_4(3) and diS-C_2-(5) from Molecular Probes; digitonin, *N*-ethylmaleimide, chlortetracycline from Serva. All other reagents were of analytical grade. 5-HETE was synthesized according to Ref. 15. [^{3}H]Arachidonic acid (190 Ci/mmol) was obtained from the Institute of Molecular Genetics, Moscow.

Results

The time course of the $[Ca^{2+}]_i$ response to thimerosal

Thimerosal (5–100 μM) induced a dose-dependent $[Ca^{2+}]_i$ rise in thymocytes. At thimerosal concentrations 5 to 10 μM $[Ca^{2+}]_i$ started to rise after 1–2 min lag and reached a plateau within 4 min, the plateau level being maintained during the following 15 min of observation (Fig. 1a). At a concentration of 10–50 μM thimerosal evoked a two-stage $[Ca^{2+}]_i$ rise, in which case the duration of the plateau shortened and it was followed by a second $[Ca^{2+}]_i$ rise (Fig. 1b,c). The higher thimerosal concentration, the earlier the second stage was initiated; and at a thimerosal concentration above 50 μM the plateau disappeared and the $[Ca^{2+}]_i$ response became apparently of one-stage character (Fig. 1d).

The $[Ca^{2+}]_i$ signals elicited during the first and second stages differed markedly in their magnitude. During the first stage $[Ca^{2+}]_i$ only rose to about 160–180 nM, while the second stage resulted in $[Ca^{2+}]_i$ rise above 1 μM, i.e., up to full saturation of quin2 with Ca^{2+}.

The time course of $[Ca^{2+}]_i$ changes presented in Fig. 1(a–d) was constantly reproduced in all the repetitive experiments ($n = 5$ for each curve), whereas the range of thimerosal concentrations which produced the two-stage $[Ca^{2+}]_i$ response (that is, the intermediate plateau, Fig. 1b,c) somewhat varied.

Sources of $[Ca^{2+}]_i$ rise

The $[Ca^{2+}]_i$ rise evoked by thimerosal sharply weakened with the decrease of external Ca^{2+} concentration and completely disappeared in the Ca^{2+}-free medium (Fig. 2) where even a slight decrease in $[Ca^{2+}]_i$ was observed (Fig. 2c). At thimerosal concentration of 20 μM the $[Ca^{2+}]_i$ response was almost not seen already at 0.15 mM $[Ca^{2+}]_o$ (Fig. 2f). This suggests a major role for Ca^{2+} influx in the $[Ca^{2+}]_i$ signal induced by thimerosal.

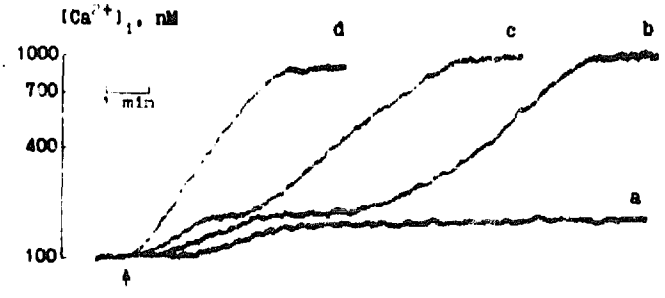

Fig. 1. Changes in $[Ca^{2+}]_i$ in rat thymocytes under the action of thimerosal (arrow) at concentrations: 5 μM (a); 15 μM (b); 50 μM (c); 100 μM (d). In this and further figures, cells were suspended in SBS at $[Ca^{2+}]_o = 1.3$ mM, if not stated otherwise. The traces are representative of five similar experiments.

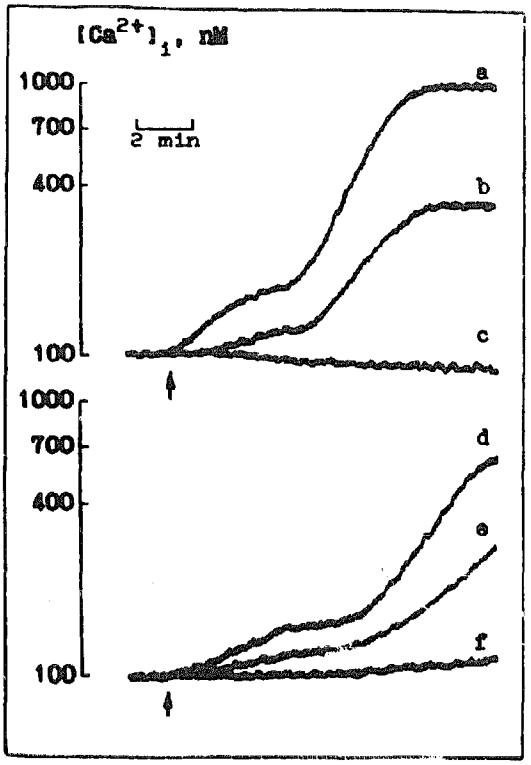

Fig. 2. $[Ca^{2+}]_i$ changes induced in rat thymocytes by 50 μM (a–c) or 20 μM (d–f) thimerosal (arrows) at different $[Ca^{2+}]_o$ levels: 1.3 mM (a,d); 0.6 mM (e); 0.15 mM (b,f); 0.15 mM $CaCl_2$ + 1 mM EGTA (c). The traces are representative of five similar experiments.

On the other hand, in permeabilized thymocytes sulfhydryl reagents *N*-ethylmaleimide and thimerosal were found to deplete intracellular Ca^{2+} stores [16]. To find out whether such depletion takes place in intact cells as well, we attempted to compare the amount of intracellular releasable Ca^{2+} in the thimerosal-treated and control cells by measuring $[Ca^{2+}]_i$ response to ionomycin in the Ca^{2+}-free medium. In cells suspended in the Ca^{2+}-free medium and treated with thimerosal, the $[Ca^{2+}]_i$ rise induced by ionomycin was markedly diminished as compared to control cells (Fig. 3A). In addition, thimerosal elicited a fall in the fluorescence of thymocytes loaded with chlortetracycline, which is known to reflect the release of membrane associated Ca^{2+} [10], i.e., intracellular Ca^{2+} mobilization (Fig. 3B). Both these results suggest that thimerosal induces depletion of intracellular Ca^{2+} stores. This depletion, however, does not elicit a $[Ca^{2+}]_i$ rise in quin2-loaded thymocytes suspended in the Ca^{2+}-free medium (Fig. 2c).

Properties of thimerosal-induced $[Ca^{2+}]_i$ signal

SH reagents have been reported to stimulate K^+/Cl^- cotransport in eukaryotic cells [7]. Therefore, we have checked the effects of K^+/Cl^- cotransport blockers furosemide and SITS on the $[Ca^{2+}]_i$ response to thimerosal. In thymocytes pretreated for 30 min with either 1 mM furosemide or 20 μM SITS, the $[Ca^{2+}]_i$

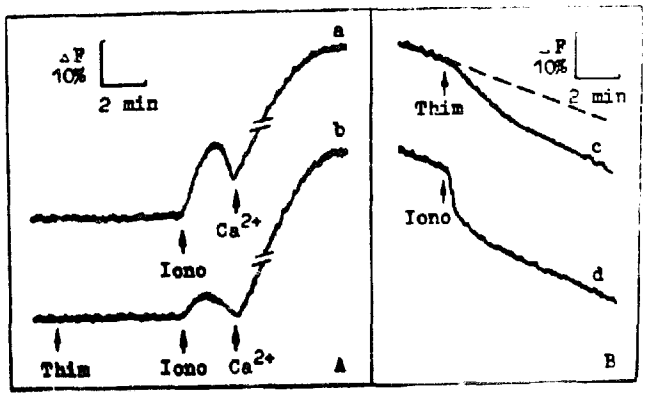

Fig. 3. Depletion of intracellular Ca^{2+} pools under the action of thimerosal. (A) Changes in the fluorescence of quin2-loaded cells upon sequential addition of ionomycin and $CaCl_2$ (a), or thimerosal, ionomycin and $CaCl_2$ (b). (B) Changes in the fluorescence of chlortetracycline-loaded cells upon addition of thimerosal (c) or ionomycin (d). Additions: 50 μM thimerosal (Thim); 1 μM ionomycin (Iono); 2 mM $CaCl_2$ (Ca^{2+}). Dashed line: untreated cells. In all the experiments thymocytes were suspended in SBS containing 0.15 mM $CaCl_2$ and 1 mM EGTA. The 100% level corresponds to the initial fluorescence intensity of quin2 or chlortetracycline-loaded cells established before addition of the agents. The traces are representative of four similar experiments.

response to thimerosal was unchanged as compared to control cells (not shown), suggesting that the $[Ca^{2+}]_i$ rise is not mediated by K^+/Cl^- cotransport activated by thimerosal.

The $[Ca^{2+}]_i$ response to thimerosal appeared electrogenic. In a depolarization medium in which K^+ was substituted for Na^+, the overall thimerosal-induced $[Ca^{2+}]_i$ rise markedly decreased, and the two separate stages became less pronounced (Fig. 4b). Substitution of choline for Na^+ did not affect thimerosal-induced $[Ca^{2+}]_i$ signal (Fig. 4a). The attenuatory action of depolarization evidences that the $[Ca^{2+}]_i$ signal is not mediated by voltage-gated Ca^{2+} channels. This conclusion is also confirmed by the finding that verapamil, a blocker of voltage-dependent Ca^{2+} channels, did not affect the $[Ca^{2+}]_i$ response to thimerosal at a concentration as high as 1 mM (not shown).

FCCP, an agent reducing cellular ATP, significantly inhibited the $[Ca^{2+}]_i$ response to thimerosal (Fig. 4B). This implies that undisturbed cellular energy metabolism is required for thimerosal to produce the rise in $[Ca^{2+}]_i$.

Thimerosal-induced influx of Mn^{2+} and Ni^{2+}

In quin2-loaded cells suspended in the standard Ca^{2+} containing medium supplemented with 1 mM Mn^{2+} or 1 mM Ni^{2+}, the fluorescence response to thimerosal changed dramatically. The initial rise in the fluorescence was followed by a rapid fluorescence drop to a level much lower than the basal one, practically to the zero level (Fig. 5a,b). This fall of the fluorescence signal is apparently caused by quenching of intracellular quin2 fluorescence [12] due to thimerosal-induced Ni^{2+} or Mn^{2+} entry. Note that neither Ni^{2+} nor Mn^{2+} changed the basal fluorescence level of quin2-loaded thymocytes (Fig. 5a,b).

It is interesting that Ni^{2+}, which is known to block Ca^{2+} channels in eukaryotic cells [17,18], failed to inhibit the initial stage of the $[Ca^{2+}]_i$ response to thimerosal (Fig. 5a). Moreover, during the first seconds after its application thimerosal stimulates $[Ca^{2+}]_i$ rise but elicits neither Mn^{2+} nor Ni^{2+} influx. Thimerosal-induced conductivity for heavy metals lagged behind the $[Ca^{2+}]_i$ signal (Fig. 5a,b). This conductivity seems to be dependent on the $[Ca^{2+}]_i$ level or the duration of thimerosal treatment. The higher $[Ca^{2+}]_i$ level (or the longer the time interval after thimerosal application), the earlier Ni^{2+} or Mn^{2+} entry was initiated (Fig. 5c).

In contrast to the effect of thimerosal observed in Ca^{2+} containing medium (Figs. 5 and 6a), in the Ca^{2+}-free medium the SH reagent elicited no substantial influx of either Mn^{2+} or Ni^{2+}. In these conditions, the gradual quenching of quin2 fluorescence in untreated cells was much more pronounced (Fig. 6, b–d), as compared to Ca^{2+} containing medium (Fig. 5). This basal fluorescence quenching may be due to heavy metal entry into the cell, or to an increased dye leak-

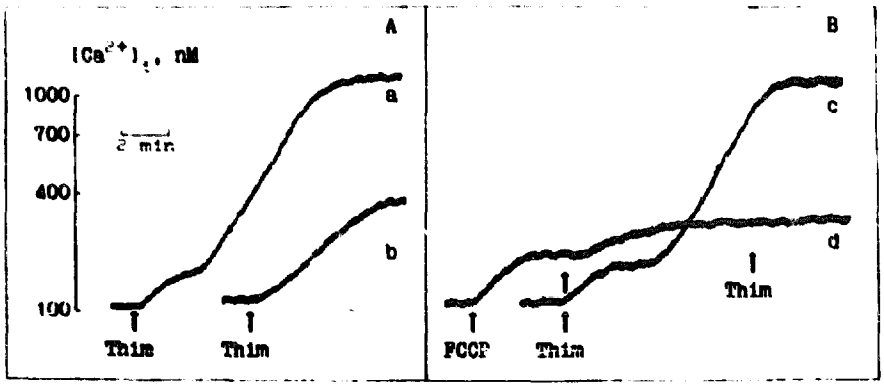

Fig. 4. $[Ca^{2+}]_i$ responses to thimerosal in a modified medium (A) and in the presence of FCCP (B). NaCl was substituted by choline chloride (a) or KCl (b). Additions: 50 μM (A) or 20 μM (B) thimerosal (Thim); 2 μM FCCP. The traces are representative of three similar experiments.

age from the cell (induced by the presence of Mn^{2+} or Ni^{2+} in the Ca^{2+}-free medium) and the subsequent quenching of extracellular quin2 by the heavy metals. In Ca^{2+}-free medium, thimerosal only produced a small, if any, additional increase in the rate of the fluorescence decay (Fig. 6, b–d), suggesting that it was no substantial entry of Mn^{2+} or Ni^{2+} into thymocytes under these conditions. We also failed to register a thimerosal-induced Ba^{2+} influx (Fig. 6e) which would have resulted in a pronounced increase in the intracellular quin2 fluorescence in the Ca^{2+}-free medium [19]. Note that Ba^{2+} is known to go through Ca^{2+} channels in eukaryotic cells [20,21].

The membrane potential changes

Thimerosal induced a marked depolarization of thymocytes, as indicated by fluorescence changes monitored with either cationic diS-C_2-(5) or anionic di-BA-C_4-(3) potential sensitive fluorescence probes (Fig. 7a,b). Similar depolarization was observed upon the action of 0.3–1 mM *N*-ethylmaleimide (data not shown). It should be noted that while diS-C_2-(5) fluorescence reflects changes in both the plasma membrane and mitochondrial membrane potential [22], di-BA-C_4-(3) may only measure the former one [13]. The similarity between the effects measured with the use of both dyes (Fig. 7a,b) evidences that the depolarization is accounted for by changes in the plasma membrane

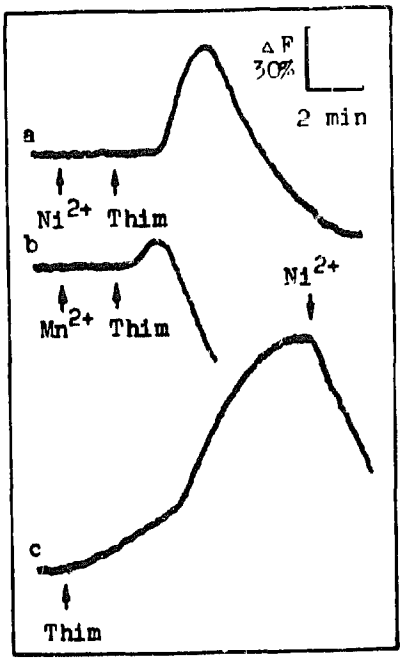

Fig. 5. Thimerosal-induced influx of Mn^{2+} and Ni^{2+}. Changes in the fluorescence of quin2-loaded thymocytes upon addition of 1 mM $NiCl_2$ (Ni^{2+}); 1 mM $MnCl_2$ (Mn^{2+}), and 40 μM thimerosal (Thim). Thymocytes were suspended in SBS containing 1.3 mM $CaCl_2$. The 100% level corresponds to the initial fluorescence intensity of quin2-loaded cells established before addition of the agents. The traces are representative of at least four similar experiments.

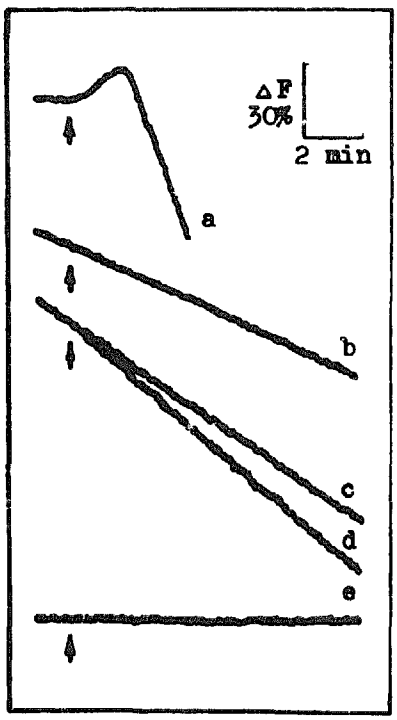

Fig. 6. Effect of thimerosal on divalent cations influx into thymocytes suspended in the presence or absence of external Ca^{2+}. Changes in the fluorescence of quin2-loaded thymocytes upon addition of 50 μM thimerosal (arrows; a,b,d,e) or in control cells (c). The cells were suspended in SBS containing: 1.3 mM $CaCl_2$ and 5 mM $NiCl_2$ (a); no added $CaCl_2$ and 5 mM $NiCl_2$ (b); no added $CaCl_2$ and 1 mM $MnCl_2$ (c,d); no added $CaCl_2$, 100 μM EGTA and 2 mM $BaCl_2$ (e). $NiCl_2$, $MnCl_2$ or $BaCl_2$ were added to cell suspension 60–90 s before addition of thimerosal. The traces are representative of at least three similar experiments.

rather than mitochondrial membrane potential. This conclusion is also confirmed by the finding that thimerosal-induced depolarization was not prevented by inhibitors abolishing the membrane potential of mitochondria, oligomycin plus rotenone (Fig. 7c).

The depolarization induced by thimerosal was also of a two-stage character (Fig. 7), its overall magnitude being about 40 mV. The same two-stage time course of the membrane potential response to thimerosal was observed in the presence of 1 mM ouabain, an inhibitor of Na^+/K^+-ATPase (not shown), thus implying that the effect of thimerosal was not mediated by Na^+/K^+-ATPase inhibition. Neither removal of Cl^- from the extracellular medium (Fig. 8a), nor application of the inhibitors of Cl^- transport furosemide (1 mM) of SITS (20 μM) (not shown) affected the changes in the membrane potential elicited by thimerosal. By contrast, in the Na^+-free medium the first stage of the membrane potential response to thimerosal disappeared (Fig. 8b) implying that this stage might result from an increased permeability for Na^+. The second stage preserved, implying that it was not mediated by Na^+ influx.

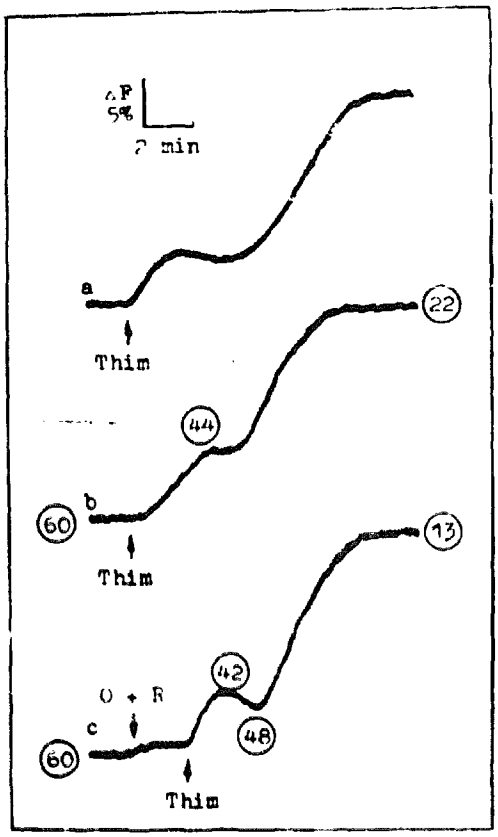

Fig. 7. Changes in di-BA-C_4-3 (a) or diS-C_3-(5) (b,c) fluorescence in the suspension of thymocytes upon addition of thimerosal (Thim, 50 μm); oligomycin (O; 2 ng/ml) plus rotenone (R; 2 μM). The 100% level corresponds to the initial fluorescence intensity of the dyes in thymocyte suspension established before the addition of agents. The traces are representative of five similar experiments. The numbers in circles indicate the calculated membrane potential values.

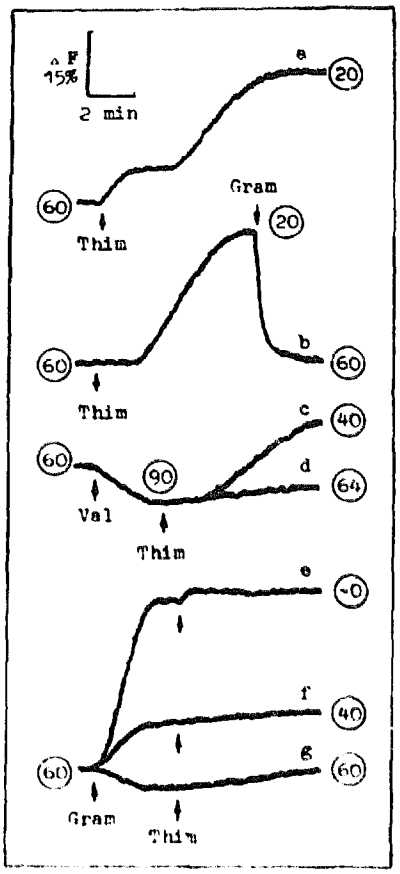

Fig. 8. Changes in the fluorescence of diS-C_3-(5) in the suspension of thymocytes upon addition of thimerosal (Thim, 50 μm); gramicidin (Gram, 1 μM) and valinomycin (Val, 5 μM). Cells were suspended in standard (c,e) or modified (a,b,d,f,g) SBS: the low-Cl^- medium (a; $[Cl^-]_o \approx 8$ mM), low-Na^+ medium (f; $[Na^+]_o \approx 16$ mM); Na^+-free medium (b,d,g; $[Na^+]_o \approx 1$ mM). The 100% level corresponds to the fluorescence intensity of diS-C_3-(5) in thymocyte suspension established before addition of the agents. The traces are representative of at least four similar experiments. The numbers in circles indicate the calculated membrane potential values.

It may be suggested that the second stage of the membrane potential response to thimerosal is accounted for by a reduced permeability for K^+. This explanation comes from the following findings. (1) Thimerosal did not change the membrane potential of cells suspended in the Na^+-free medium and treated with K^+ ionophore valinomycin (Fig. 8c,d) or with the monovalent cation channel former gramicidin (Fig. 8g). (2) Depolarization induced by thimerosal in Na^+-free medium was nearly eliminated by subsequent addition of either gramicidin (Fig. 8b) or valinomycin (not shown). (3) Thimerosal did not induce membrane potential changes in cells exposed to gramicidin in either standard or low Na^+ medium (Fig. 8e,f).

Removal of Ca^{2+} from the medium did not change the overall magnitude of the membrane potential response to thimerosal (Fig. 9a,b), hence the depolarization is not mediated by Ca^{2+} rise. However, the time course of the membrane potential response to the SH reagent became modified. In the absence of extracellular Ca^{2+} the membrane potential response developed faster as the intermediate plateau disappeared (or was

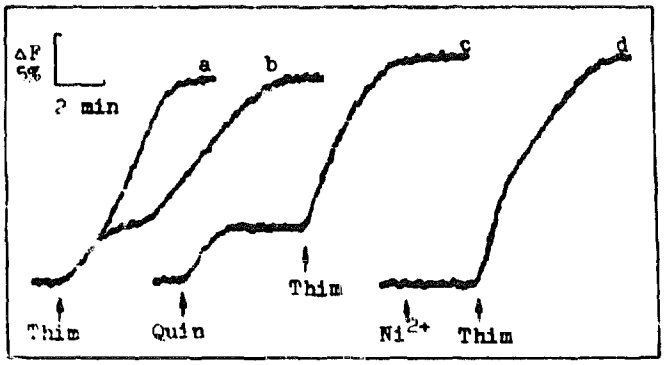

Fig. 9. Changes in the fluorescence of diS-C_3-(5) in the suspension of thymocytes upon addition of thimerosal (Thim, 50 μM); quinine (Quin, 0.25 mM) and $NiCl_2$ (Ni^{2+}, 5 mM). Cells were suspended in SBS containing 1.3 mM $CaCl_2$ (b–d) or 0.1 mM $CaCl_2$ + 1 mM EGTA (a). The 100% level corresponds to the fluorescence intensity of diS-C_3-(5) in thymocyte suspension established before addition of the agents. The traces are representative of four similar experiments.

significantly shortened) (Fig. 9a). It has been shown earlier [10,23] that a $[Ca^{2+}]_i$ rise in thymocytes results in the induction of Ca^{2+}-activated K^+ conductivity, i.e., hyperpolarization. Such a hyperpolarization induced by thimerosal may interfere with the depolarization effect of the SH reagent, and this may explain, at least partially, the appearance of the first, intermediate, plateau in the membrane potential response to thimerosal. In accordance with this assumption is the observation that the intermediate plateau also disappeared in the presence of quinine (the response to thimerosal became faster and one-stage, Fig. 9c), Ni^{2+} (Fig. 9d), or in the presence of 35 mM KCl in the external medium (not shown). It is known [10] that Ca^{2+}-dependent K^+ permeability in thymocytes is inhibited by quinine (which accounts for the slight depolarization exerted by this agent, Fig. 9c) or by elevated $[K^+]_o$. On the other hand, Ni^{2+} is an inhibitor of Ca^{2+} channels [17,18], and its annulation of the intermediate plateau of the membrane potential response to thimerosal may be explained by inhibition of thimerosal-induced Ca^{2+} entry and hence of Ca^{2+}-activated K^+ conductivity.

Involvement of AA metabolites

We have recently found that AA metabolites, probably LO products, contribute to the formation of ionic signals in thymocytes [24,25]. In macrophages, thimerosal has been shown to induce AA release and significantly stimulate LO [26]. Therefore, we tested the involvement of AA and its metabolites in the changes in ion transport induced by thimerosal. Thimerosal was found to significantly stimulate AA release from thymocytes (Fig. 10). Exogenous AA, its metabolites, and inhibitors of AA metabolism appeared to exert diverse effects on thimerosal-induced changes in $[Ca^{2+}]_i$ and membrane potential of thymocytes. The most pronounced effect on the $[Ca^{2+}]_i$ response to thimerosal was produced by NDGA, an inhibitor of LO, in particular 5-LO [24]. The $[Ca^{2+}]_i$ response to thimerosal was significantly retarded by NDGA, and the delayed $[Ca^{2+}]_i$ signal became one-state (Fig. 11). Indomethacin (5–15 μM), a cyclooxygenase inhibitor, did not affect the $[Ca^{2+}]_i$ response to thimerosal (not shown). These data may suggest the involvement of LO product(s) in $[Ca^{2+}]_i$ signal induced by thimerosal.

Exogenous AA was shown to modulate synthesis of LO products induced by thimerosal in macrophages [26]. As seen in Fig. 11, exogenous AA and its LO product 5-HETE both exerted inhibitory effects on the $[Ca^{2+}]_i$ response to thimerosal. If applied after thimerosal, neither AA nor 5-HETE eliminated the $[Ca^{2+}]_i$ signal evoked by thimerosal (not shown). The observed modulation by AA and 5-HETE of thimerosal-induced changes in $[Ca^{2+}]_i$ also supports

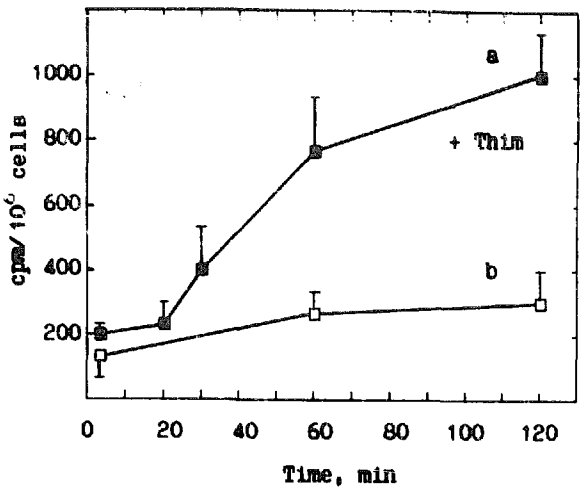

Fig. 10. The time course of [^{3}H]arachidonic acid release from thimerosal-treated (a) and untreated (b) thymocytes. In curve (a), 3 ml of [^{3}H]arachidonic-labeled thymocytes ($1.5 \cdot 10^7$ thymocytes/ml) were treated with 20 μM thimerosal. At times indicated the net radioactivity released in the supernatant was measured as described in Materials and Methods. Each point is the mean of quadruplicate determinations.

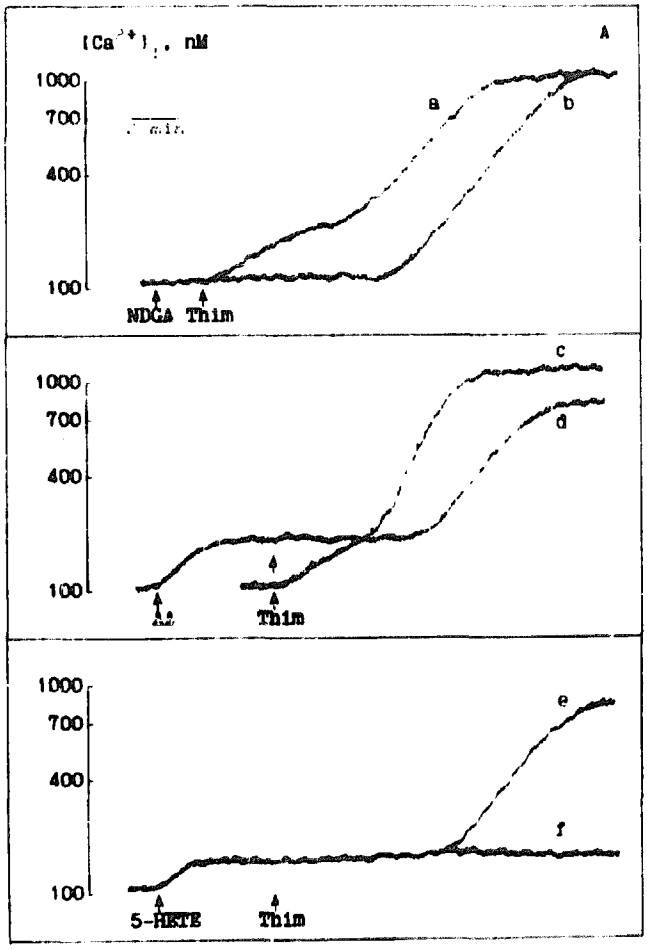

Fig. 11. $[Ca^{2+}]_i$ responses to thimerosal in the absence (a,c) and presence (b,d,e,f) of NDGA (b); AA (d); 5-HETE (e,f). Additions: 10 μM thimerosal (a,b,f); 20 μM thimerosal (c,e,d); 15 μM NDGA (b); 3 μM AA (d); 30 μM HETE (e,f). The traces are representative of three similar experiments.

the idea that these changes may be mediated by some AA metabolite(s).

Both AA and 5-HETE were found to induce a marked depolarization (Fig. 12). The effect was not eliminated by oligomycin plus rotenone, the inhibitors abolishing the mitochondrial membrane potential (not shown). As in the case of thimerosal (Figs. 8, 9), the depolarization induced by AA and 5-HETE was preserved in the Ca^{2+}-free or Na^+-free medium, or in the presence of ouabain (data not shown), but it was eliminated by agents increasing K^+ permeability, valinomycin or nigericin (Fig. 12a,b), or in a K^+-rich medium. Thus, the depolarization induced by AA and 5-HETE is similar in its characteristics to that induced by thimerosal, and it may similarly be accounted for by a reduced permeability for K^+.

In the presence of AA or 5-HETE, the membrane potential response to thimerosal was modified: the first, slow stage disappeared and the overall magnitude decreased (Fig. 12d,e). The result of successive applications of AA and thimerosal varied depending on the sequence of the additions of the agents (Fig. 12d,f). The same was observed for 5-HETE (not shown). The found cross-influence of AA and thimerosal (or 5-HETE and thimerosal) on their effects on thymocyte membrane potential may suggest that thimerosal-induced depolarization is mediated by some AA metabolite(s).

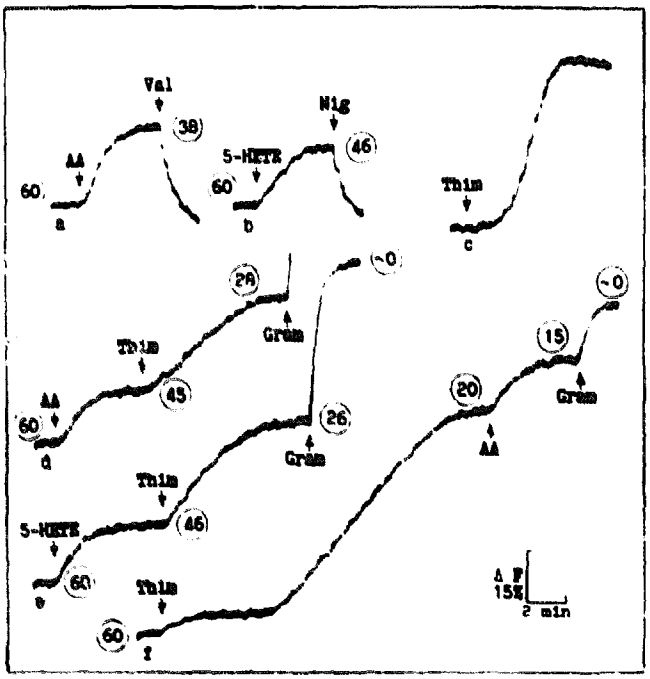

Fig. 12. Changes in the fluorescence of diS-C_3-(5) in the suspension of thymocytes upon successive additions of AA, 5-HETE, thimerosal and ionophores: valinomycin, nigericin, gramicidin. Additions: 15 μM AA (a); 6 μM AA (d,f); 30 μM 5-HETE (b,e); 10 μM thimerosal (Thim, c–f); 5 μM valinomycin (Val, a); 2 μM nigericin (Nig, b); 1 μM gramicidin (Gram, d–f). In trace (e), 15 μM NDGA was added to the cells 2 min before addition of thimerosal. The 100% level corresponds to the initial fluorescence intensity of the dye in thymocyte suspension established before the addition of agents. The traces are representative of three similar experiments. The numbers in circles indicate the calculated membrane potential values.

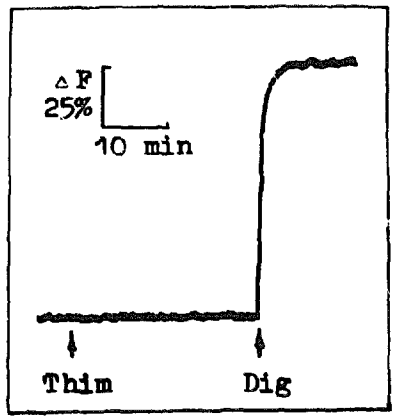

Fig. 13. Changes in the fluorescence of 30 μM ethidium bromide in the suspension of thymocytes upon addition of thimerosal (Thim, 50 μm) and digitonin (Dig, 25 μM). The 100% level corresponds to the fluorescence intensity established after addition of digitonin.

In the presence of NDGA, the membrane potential response to thimerosal developed much faster (Fig. 12, curve e). The fluorescence of diS-C_3-(5) was somewhat quenched by NDGA which made difficult quantitative estimates of membrane potential. Indomethacin (5–15 μM) did not affect the pattern of membrane potential changes induced by thimerosal (not shown).

Cell viability in the presence of thimerosal

As measured by Trypan blue exclusion, the viability of thymocytes treated with thimerosal for 20 min was the same as observed for control cells (> 95%). Ethidium bromide fluorescence in thymocyte suspension was also unchanged upon addition of thimerosal though it sharply increased upon subsequent thymocyte permeabilization with digitonin (Fig. 13). Hence, at least for 20 min application, thimerosal does not induce thymocyte plasma membrane permeability for molecules of ethidium bromide size. Incubation with 5–50 μM thimerosal for 24 h resulted, however, in pronounced cell death: the percentage of thymocytes that excluded Trypan blue dropped to 34 ± 6% as compared to 84 ± 8% for control cells (n = 3).

Discussion

The results obtained indicate that the activity of various ion transporting systems in thymocytes is markedly changed upon the action of thimerosal. First of all, thimerosal has been found to raise $[Ca^{2+}]_i$, and not only in thymocytes [9–11] but in macrophages [26] and platelets [1] as well. In earlier studies on thymocytes, however, the $[Ca^{2+}]_i$ changes were only monitored during several minutes, and only the first stage of

the $[Ca^{2+}]_i$ response to thimerosal has been registered [9–11]. The present work demonstrates that during the second stage $[Ca^{2+}]_i$ increases up to 1 μM, even at thimerosal concentration as low as 10 μM.

Thimerosal induces both Ca^{2+} influx and depletion of intracellular Ca^{2+} pools (Figs. 1–3). The intracellular Ca^{2+} mobilization may be accounted for by thimerosal inhibition of Ca^{2+}-ATPase of the endoplasmic reticulum. Indeed, in experiments on permeabilized thymocytes we have found that thimerosal induces depletion of Ca^{2+} pools [16]. It is worth noting that the SH reagent did not prevent Ca^{2+} release from the thymocyte endoplasmic reticulum induced by inositol 1,4,5-trisphosphate [16].

Despite the evidence for intracellular Ca^{2+} mobilization, we failed to observe $[Ca^{2+}]_i$ response to thimerosal in the Ca^{2+}-free medium (Figs. 2, 3). This may be explained by at least two reasons. First, $[Ca^{2+}]_i$ rise in Ca^{2+}-free medium may be dampen because of quin2 buffering. A very slight increase in $[Ca^{2+}]_i$ due to intracellular Ca^{2+} mobilization has recently been observed [11] in thymocytes loaded with fura-2, a dye with a much lower buffering capacity. Second, Ca^{2+} may be released from intracellular stores rather slow, so that Ca^{2+} mobilized from intracellular stores might be extruded from the cell without any significant $[Ca^{2+}]_i$ rise in quin2-loaded cells [28]. Indeed, the drop in chlortetracycline fluorescence elicited by thimerosal is much slower than that induced by ionomycin (Fig. 3B).

In general, Ca^{2+} pumps are known to be SH-dependent enzymes [26,27]. So, Ca^{2+}-ATPase of thymocyte plasma membrane could also serve as a target for thimerosal. However, in the presence of thimerosal the $[Ca^{2+}]_i$ response to ionomycin in the Ca^{2+}-free medium remained transient (Fig. 3a,b), which implies that the plasma membrane Ca^{2+}-ATPase is not impaired by the SH reagent. Thus, an increased Ca^{2+} influx may be considered as the main source of thimerosal-induced $[Ca^{2+}]_i$ rise.

The nature of the two-stage time course of the $[Ca^{2+}]_i$ response to thimerosal (Fig. 1) is not clear. The Ca^{2+}-transporting system(s) responsible for the first stage of $[Ca^{2+}]_i$ rise is not inhibited by Ni^{2+} and does not transfer Mn^{2+} (Fig. 5). This distinguishes it from Ca^{2+} channels found in the plasma membrane of nonexcitable cells [17,18]. During the second stage of the Ca^{2+} response to thimerosal there arises a conductivity for several divalent cations (Fig. 5). Most unusual, thimerosal, as well as 5-HETE (Fig. 11), induces permeability not only for Ca^{2+} and Mn^{2+} but for Ni^{2+} as well, which is distinct from the known types of Ca^{2+} channels [17,18]. It is also important that the induction of the conductivity for the divalent cations is $[Ca^{2+}]_o$ dependent (Fig. 6). The initial $[Ca^{2+}]_i$ rise seems to promote the subsequent divalent ion entry (Figs. 5 and 6).

By contrast, the external Ca^{2+} is not required for thimerosal-induced changes in monovalent ion fluxes resulting in depolarization (Figs. 7–9). Depolarization appears to be mediated by changes in the plasma membrane permeabilities for both Na^+ and K^+. It is of interest that thimerosal may regulate the ion fluxes in opposite directions, increasing the permeability for Ca^{2+} and Na^+ and decreasing it for K^+.

Thimerosal markedly stimulates AA release from thymocytes (Fig. 10); a similar effect has been observed in a number of cells [26]. The results obtained (Figs. 11 and 12) enable to suggest the involvement of AA metabolite(s), probably LO product(s), in the effects of thimerosal on the transport of mono- and divalent cations. This is evidenced by the following findings. (1) The $[Ca^{2+}]_i$ and membrane potential responses to thimerosal are markedly affected by NDGA, exogenous AA and 5-HETE. (2) Exogenous AA and 5-HETE produce changes in ion transport similar to those exerted by thimerosal, namely, a rise in $[Ca^{2+}]_i$ and depolarization (this work), and a decrease in pH_i [11,24].

AA metabolite(s) that may mediate the effects of thimerosal remains to be identified. The involvement of cyclooxygenase products seems improbable, since the cyclooxygenase inhibitor indomethacin did not affect the changes in $[Ca^{2+}]_i$ and membrane potential induced by thimerosal. The experiments with sequential additions of AA (or 5-HETE) and thimerosal (Fig. 11) suggest that neither AA nor 5-HETE themselves mediate the $[Ca^{2+}]_i$ response to thimerosal, as it is inhibited by these agents. It might be thought that AA or 5-HETE change the spectrum of AA metabolites produced by thimerosal [26]. The facts that thimerosal-induced changes in ion transport are modulated by AA, its metabolite and the LO inhibitor, and the dependence of the $[Ca^{2+}]_i$ response to thimerosal on cellular ATP level (Fig. 4c), make it doubtful that the action of thimerosal on thymocyte plasma membrane permeabilities for mono- and divalent cations is due solely to some nonspecific effect of the sulfhydryl reagent.

In platelets, thimerosal was found to mimic the effects of receptor-interacting agonists, inducing secretion and aggregation [1]. In thymocytes, however, thimerosal failed to cause proliferation and, moreover, it inhibited proliferation stimulated by PHA [11]. The idea that thimerosal might induce proliferation came from the observation [11] that it exerted $[Ca^{2+}]_i$ and pH_i changes similar to those elicited by common proliferatory stimuli. However, typical Ca^{2+}-mobilizing receptor-interacting agonists only produce a transient and moderate rise in $[Ca^{2+}]_i$ which critically distinguishes them from thimerosal. As found in the present work, thimerosal induces not proliferation but cell death, which may be accounted for by the high steady

levels of $[Ca^{2+}]_i$ established in thymocytes treated with the SH reagent. In addition, thimerosal induces a marked depolarization of thymocytes which also distinguishes it from proliferatory stimuli. The mechanism of thimerosal-induced cell death is the subject of our further studies.

References

1 Hecker, M., Brüne, B., Decker, K. and Ullrich, V. (1989) Biochem. Biophys. Res. Commun. 159, 961–968.
2 De Nucci, G., Gryglewski, R.J., Warner, T.D. and Vane, J.R. (1988) Proc. Natl. Acad. Sci. USA 85, 2334–2338.
3 Boobis, A.R., Fawthrop, D.J. and Davies, D.S. (1989) Trends Pharm. Sci. 10, 277–281.
4 Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. (1989) Trends Pharm. Sci. 10, 281–285.
5 Gardner, P. (1989) Cell 59, 15–20.
6 Grinstein, S. and Dixon, S.J. (1989) Physiol. Rev. 69, 417–481.
7 Lauf, P.K. (1984) J. Membr. Biol. 77, 57–62.
8 Grinstein, S., Cohen, S. and Rothstein, A. (1985) Biochim. Biophys. Acta 812, 213–222.
9 Gukovskaya, A.S., Zinchenko, V.P. and Mantsygin, Yu.A. (1987) Ukrainskii Biokhim. Zhurnal 59, 39–42 (in Russian).
10 Gukovskaya, A.S. and Zinchenko, V.P. (1990) Sov. Sci. Rev. D. Physic. Chem. Biol. (Harwood Academic Publishers) 10, 1–98.
11 Martin, F., Gualberto, A., Sorbino, F. and Pintado, E. (1991) Biochim. Biophys. Acta 1091, 110–14.
12 Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. (1983) J. Biol. Chem. 258, 4876–4882.
13 Wilson, H.A. and Chused, T.M. (1985) J. Cell Physiol. 125, 72–81.
14 Grinstein, S., Goetz, J.D. and Rothstein, A. (1984) J. Gen. Physiol. 84, 565–584.
15 Balswin, T.E., Dewies, D.J., Hughes, J. and Gulteridge, N.J.A. (1979) J. Chem. Soc. (Perkin Trans. 1) 1, 115–120.
16 Gukovskaya, A.S., Zinchenko, V.P., Petrynyaka, V.V., Khodorov, B.I. and Evtodienko, Yu.B. (1986) Eur. J. Biochem. 161, 249–256.
17 Merritt, J.E., Jacob, R. and Hallam, T.J. (1989) J. Biol. Chem. 264, 1522–1527.
18 Chow, S.C. and Jondal, M. (1990) J. Biol. Chem. 265, 902–907.
19 Gukovskaya, A.S., Zinchenko, V.P. and Khodorov, B.I. (1987) Biol. Membr. 4, 923–930 (in Russian).
20 Kostyuk, P.C. (1984) Physiol. Rev. 64, 435–454.
21 Hess, P. and Tsien, R. (1984) Nature 309, 453–456.
22 Wilson, H.A., Seligman, B.E. and Chused, T.M. (1985) J. Cell Physiol. 125, 61–71.
23 Grinstein, S., Cohen, S., Sarkadi, B. and Rothstein, A. (1983) J. Gen. Physiol. 116, 352–362.
24 Gukovskaya, A.S., Arias Pulido, H. and Zinchenko, V.P. (1989) FEBS Lett. 244, 461–464.
25 Gukovskaya, A.S., Arias Pulido, H., Petrunyaka, V.V., Zinchenko, V.P. and Bezuglov, V.V. (1990) Cell Calcium 11, 539–546.
26 Kaever, V., Goppelt-Strübe, M. and Resch, K. (1988) Prostaglandins 35, 885–903.
27 Humes, J.L., Sadowski, S., Galavage, M. et al. (1983) Biochem. Pharmacol. 32, 2319–2322.
28 Zinchenko, V.P., Nikiforov, E.I., Kim, Yu.A. and Gukovskaya, A.S. (1992) FEBS Lett. (in press).
29 Nicotera, P., Moore, M., Mirabeli, F., Bellomo, G. and Orrenius, S. (1985) FEBS Lett. 181, 149–153.
30 Bellomo, G., Mirabelli, F., Richelmi, P. and Orrenius, S. (1983) FEBS Lett. 163, 136–139.