The Thiol Reagent, Thimerosal, Evokes Ca\textsuperscript{2+} Spikes in HeLa Cells by Sensitizing the Inositol 1,4,5-Trisphosphate Receptor*

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The thiol reagent, thimerosal, has been shown to cause an increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) in several cell types, and to cause Ca\textsuperscript{2+} spikes in unfertilized hamster eggs. Using single cell video-imaging we have shown that thimerosal evokes repetitive Ca\textsuperscript{2+} spikes in intact Fura-2-loaded HeLa cells that were similar in shape to those stimulated by histamine. Both thimerosal- and histamine-stimulated Ca\textsuperscript{2+} spikes occurred in the absence of extracellular (Ca\textsuperscript{2+}o), suggesting that they result from mobilization of Ca\textsuperscript{2+} from intracellular stores. Whereas histamine stimulated formation of inositol phosphates, thimerosal, at concentrations that caused sustained Ca\textsuperscript{2+} spiking, inhibited basal and histamine-stimulated formation of inositol phosphates. Thimerosal-evoked Ca\textsuperscript{2+} spikes are therefore not due to the stimulated production of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}). The effects of thimerosal on Ca\textsuperscript{2+} spiking were probably due to alkylation of thiol groups on intracellular proteins because the spiking was reversed by the thiol-reducing compound diithiothreitol, and the latency between addition of thimerosal and a rise in [Ca\textsuperscript{2+}]i was greatly shortened in cells where the intracellular reduced glutathione concentration had been decreased by preincubation with dL-buthionine (S,R)-sulfoximine. In permeabilized cells, thimerosal caused a concentration-dependent inhibition of Ca\textsuperscript{2+} accumulation, which was entirely due to inhibition of Ca\textsuperscript{2+} uptake into stores because thimerosal did not affect unidirectional 44Ca\textsuperscript{2+} efflux from stores preloaded with 44Ca\textsuperscript{2+}. Thimerosal also caused a concentration-dependent sensitization of InsP\textsubscript{3}-induced Ca\textsuperscript{2+} mobilization: half-maximal mobilization of Ca\textsuperscript{2+} stores occurred with 161 ± 20 nM InsP\textsubscript{3} in control cells and with 62 ± 5 nM InsP\textsubscript{3} after treatment with 10 μM thimerosal. We conclude that thimerosal can mimic the effects of histamine on intracellular Ca\textsuperscript{2+} spiking without stimulating the formation of InsP\textsubscript{3} and, in light of our results with permeabilized cells, suggest that thimerosal stimulates spiking by sensitizing cells to basal InsP\textsubscript{3} levels.

In many cell types, hormones that activate phosphoinosi-1

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1 The abbreviations used are: [Ca\textsuperscript{2+}]i, intracellular free [Ca\textsuperscript{2+}]; BSO, dL-buthionine (S,R)-sulfoximine; Ca\textsuperscript{2+o}, extracellular Ca\textsuperscript{2+}; EM, extracellular medium; HEPES, N-(2-hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid); PIPES, piperoxene-N,N'-bis[2-ethanesulfonic acid]; t-BHP, tert-butyl hydroperoxide; InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; DTT, dithiothreitol.
Thimerosal (sodium ethylmercurithiosalicylate), a thiol alkylating agent, mobilizes Ca\(^{2+}\) from intracellular stores in leukocytes (Hartsczmmann et al., 1989) and platelets (Hecker et al., 1989), and mimics the Ca\(^{2+}\) spikes that follow fertilization of hamster (Swann, 1991) and mouse\(^{\ast}\) eggs. The mechanism underlying these actions of thimerosal is unknown. In the present study we have used human HeLa carcinoma cells to investigate the mechanism of thimerosal-induced Ca\(^{2+}\) spiking.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were grown in minimal essential medium supplemented with 5% mixed serum (50% newborn calf, 50% fetal calf), 2 mM glutamine, 60 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin. Cells were grown either on plastic dishes or, for single cell imaging studies, on glass coverslips (22 mm diameter, Chance Proper Ltd, Smethwick, Warley, United Kingdom) in a humidified atmosphere (5% CO\(_2\), 95% air) at 37 °C and fed daily.

\(\text{Ca}^{2+}\) Flux Measurements—HeLa cells were removed from dishes by treatment for 2 min with pancreatin (2.5 mg/ml), centrifuged (1000 \(\times\) g, 5 min), and resuspended at a density of 6 \(\times\) 10\(^5\) cells/ml in Ca\(^{2+}\)-free medium. Cells were permeabilized by treatment with saponin (0.15 mg/ml; 10 min at 37 °C), centrifuged (1900 \(\times\) g, 5 min), washed once, and finally resuspended at 6 \(\times\) 10\(^5\) cells/ml in cytosol-like medium. Cytosol-like medium had the following composition (mM): KCl, 140; NaCl, 20; MgCl\(_2\), 2; EGTA, 1; CaCl\(_2\), 200 \mu\M; PIPES, 20; pH 6.8. The free Ca\(^{2+}\) of cytosol-like medium measured with Fura-2 was 105 nM. \(\text{Ca}^{2+}\) (2 \(\mu\)M/ml) and the mitochondrial inhibitors antimycin (10 \muM) and oligomycin (10 \muM) were added to the permeabilized cells, and they were then stored on ice for up to 2 h before use. Experiments were initiated by warming cells to 37 °C for 2 min, and \(\text{Ca}^{2+}\) accumulation was then stimulated by addition of ATP (1.5 mM); after 5 min the cells had loaded to steady state.

To examine the effect of thimerosal on the mobilization of \(\text{Ca}^{2+}\) by InsP\(_3\), permeabilized HeLa cells were loaded to steady-state (5 min) with \(\text{Ca}^{2+}\) (2 \(\mu\)M/ml) in the presence or absence of thimerosal and then added to InsP\(_3\). Incubations were terminated by rapid filtration through Whatman GF/C filters (Boothman et al., 1992). Concentration-response curves were fitted to a logistic equation.

\[
R = 100\% - \frac{100\%}{1 + ([\text{InsP}_3]/EC_{50})n} 
\]

(Eq. 1)

\(R\) = response (% mobilization of InsP\(_3\)-sensitive \(\text{Ca}^{2+}\) stores); \text{EC}_{50} = concentration of InsP\(_3\) causing half-maximal mobilization of InsP\(_3\)-sensitive \(\text{Ca}^{2+}\) stores; \(n\) = an empirical value equivalent to the Hill coefficient.

The effect of thimerosal on unidirectional \(\text{Ca}^{2+}\) efflux from permeabilized cells was examined by loading the cells to steady-state with \(\text{Ca}^{2+}\), and then simultaneously adding thimerosal and removing the ATP by addition of glucose (10 mM) and hexokinase (25 units/ml) (Taylor and Potter, 1980). The incubations were stopped at 10-s intervals by rapid filtration. The results were fitted to a single exponential decay equation using the GraphPAD Inplot curve-fitting program (GraphPAD software, San Diego) by a nonlinear least-squares procedure.

In all of these experiments, InsP\(_3\), metabolism was negligible because even after a 5-min incubation of cells with trace amounts of \([\text{H}]\text{ins}(1,4,5)\text{P}_3\) more than 95% was recovered unchanged.

**Fura-2 Measurements of Intact Cells**—For single cell measurements of \([\text{Ca}^{2+}]\), cells grown on glass coverslips were washed with extracellular medium (EM) containing (mM): NaCl, 121; KCl, 5.4; MgCl\(_2\), 0.8; CaCl\(_2\), 1.8; NaHCO\(_3\), 11; glucose, 5.5; HEPES, 25; pH 7.4. They were then loaded with Fura-2 by incubation with 1 \muM Fura-2 acetoxymethyl ester (Fura-2/AM) (Molecular Probes Inc.) for 30 min at room temperature (20 °C) and then washed in EM. A coverslip was mounted at room temperature on the stage of a Nikon diaphot inverted epifluorescence microscope. Fluorescent images were obtained by alternate excitation at 340 and 380 nm (40 nm at each wavelength) using either twin xenon arc lamps (Spx Industries Inc.) or a rotating filter wheel (Magical, Joyce Loeb). The emission signal was collected at 510 nm using an intensified charge-coupled device video camera (Photonic Science) and the digitized signals stored and processed as described previously (O'Sullivan et al., 1989). The fluorescence ratio was obtained at video rate and filtered with a time constant of 200 ms (Spex System) or at 3-s intervals (Joyce Loeb System).

All experiments involving Fura-2 shown in the present study were performed at 20 °C because, although Fura-2/AM can be loaded into HeLa cells at 37 °C, the hydrophilic dye is rapidly lost from the cells. This loss of Fura-2 at 37 °C is slowed, but not completely inhibited, by the anion transport inhibitor, sulfinpyrazone; by loading cells at 20 °C and then warming them to 37 °C in the presence of sulfinpyrazone (100 \muM), it was possible to observe similar responses to both histamine and thimerosal at 37 °C.

**Measurement of Inositol Phosphates Production**—HeLa cells were grown in inositol-free minimal essential medium, supplemented with 5% dialyzed mixed serum, \text{mvo} \text{[H]}inositol (100 \muM/mmol; 1 \muM/ml), and DL-buthionine (S,R)-sulfoximine (BSO, 1 mM) for 24 h. The cells were removed from dishes, resuspended in EM, and after 30 min, LiCl (50 mM) was added. After a further 10 min, the cells were stimulated with histamine or thimerosal. The incubations were terminated by rapidly heating the cells. Cell debris was pelleted by centrifugation (2000 \(\times\) g, 10 min), and the supernatant was loaded onto an anion exchange column (AG 1-2X8, formate form, 200-400 mesh, Bio-Rad). \[\text{[H]}\text{inositol and \{H\}glycerophosphoinositol were eluted with 8 ml of 50 \muM ammonium formate/5 mM NaBO}_3, \text{InsP}_2 with 8 ml of 0.2 \muM ammonium formate, 0.1 \muM formic acid, \text{InsP}_3 with 8 ml of 0.5 \muM ammonium formate, 0.1 \muM formic acid, and \text{InsP}_4 were together eluted with 8 ml of 1.25 \muM ammonium formate, 0.1 M formic acid. The activity in each inositol phosphate fraction was expressed as a fraction of the total cell labeling.

**Materials**—Fura-2/AM was from Molecular Probes (Eugene, OR). Cell culture materials were from Gibco. Histamine chloride, hexokinase, monochiorobimane, BSO, and saponin were from Sigma. ATP was from Boehringer. InsP\(_3\) was from Dr. Robin Irvine (Babraham, United Kingdom). \text{Ca}^{2+}, \text{H}[^{3}H]\text{inositoli, and \{H\}InsP}_{3}\text{ were from Amersham.}

**RESULTS**

**Histamine- and Thimerosal-induced \text{Ca}^{2+}\) Spikes**—Histamine and thimerosal induced \text{Ca}^{2+}\) spikes in single HeLa cells in the presence or absence of extracellular \text{Ca}^{2+} (Ca\(_{\text{ext}}\)) (Figs. 1 and 2). With histamine-stimulated cells, the first spike usually had a different shape and was larger than successive spikes, which had similar amplitudes and were more consistent in their shape. The frequency of spiking was lower at 1 \muM histamine (Fig. 1, c and d), compared to the response at 25 \muM (Fig. 1, a and b). When Ca\(_{\text{ext}}\) was reduced below 1 \muM, histamine (25 \muM, Fig. 1e) evoked a few spikes only, but spiking was resumed when Ca\(_{\text{ext}}\) was elevated to 1.8 mM. In contrast to the regular spiking behavior observed with histamine, the shape and pattern of the \text{Ca}^{2+}\) spikes induced by thimerosal were much more variable, and were dependent upon the duration of thimerosal application, its concentration, and the presence of Ca\(_{\text{ext}}\) (Fig. 2). For example, in the presence of Ca\(_{\text{ext}}\), continuous perfusion of cells with 100 \muM thimerosal stimulated an increased \text{Ca}^{2+}\) in all of 53 cells examined, but the \text{Ca}^{2+}\) spikes progressively broadened and the basal \text{Ca}^{2+}\) rose until individual spikes were no longer distinguishable (Fig. 2a). The same perfusion protocol in the absence of Ca\(_{\text{ext}}\), however, evoked a response that was similar to that triggered by histamine, namely repetitive \text{Ca}^{2+}\) spikes of very similar shape and no increase in the basal \text{Ca}^{2+}\) (Fig. 2b). Another consistently successful protocol whereby the response to thimerosal closely resembled the effects of histamine on \text{Ca}^{2+}\) spiking was to perfuse cells with 100 \muM th-
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Fig. 1. Histamine-induced Ca²⁺ spikes in single HeLa cells. The arrows denote the onset of perfusion of cells with maximal and submaximal histamine concentrations: a, b, and e, 25 μM; c and d, 1 μM. Ca²⁺ (1.8 mM) was included in the extracellular medium throughout the perfusion period in traces a–d and for only the period shown by the heavy bar in trace e. In most single cell recordings in this and subsequent figures, the changes in [Ca²⁺]ᵢ are shown as the 340 nm/380 nm fluorescence ratio (see "Experimental Procedures"). Trace a shows the calibrated change in [Ca²⁺]ᵢ for a typical HeLa cell. In trace e, Ca²⁺ spikes from two individual HeLa cells stimulated with histamine (25 μM) have been superimposed to make clear that Ca²⁺ spiking ceases in the absence of Ca²⁺ and is rapidly recovered when Ca²⁺ is restored.

The averaged shapes of thimerosal- and histamine-stimulated Ca²⁺ spikes are shown in Fig. 4. These results show that thimerosal-evoked repetitive Ca²⁺ spikes had the same amplitudes as those evoked by histamine, with no change in the resting [Ca²⁺]. However, both the rising and recovery phases of thimerosal-evoked Ca²⁺ spikes were slower than for histamine-evoked Ca²⁺ spikes. Correspondingly, the thimerosal-evoked Ca²⁺ spikes were broader than those evoked by histamine, with half-height widths of 20 and 10 s, respectively. The different kinetics are particularly evident when the rates of [Ca²⁺]ᵢ change are plotted either against time (Fig. 4, aii and biii) or against [Ca²⁺] (Fig. 4, aii and biii). The phase diagrams (Fig. 4, aii and biii), clearly indicate that for both histamine- and thimerosal-stimulated spikes, the rate of [Ca²⁺]ᵢ rise is faster than the rate of recovery.

A brief incubation (50 s) with 100 μM thimerosal evoked Ca²⁺ spikes in 12 out of 29 cells; in 50% of these cells the spiking ceased within 6 min of the removal of thimerosal (not shown). Longer perfusions with 100 μM thimerosal, which caused the sustained increase in [Ca²⁺], (Fig. 2a), were not reversed after removal of thimerosal (results not shown). Both the spiking behavior initiated by brief incubation with thimerosal and the otherwise irreversible sustained increase in [Ca²⁺], that followed prolonged incubation with 100 μM thimerosal were rapidly reversed by addition of dithiothreitol.

Fig. 2. Effect of thimerosal on [Ca²⁺] in single HeLa cells in Ca²⁺-free and Ca²⁺-containing EM. The 3 cells in a were perfused with EM supplemented with 100 μM thimerosal. Cells in b were perfused with Ca²⁺-free EM ([Ca²⁺]₀ < 1 μM) containing 100 μM thimerosal or 25 μM histamine as indicated.

Fig. 3. Repetitive Ca²⁺ spikes in single thimerosal-stimulated HeLa cells. Cells in Ca²⁺-containing EM were perfused with 100 μM thimerosal for 12 min and then continuously perfused with 1 μM thimerosal. Results from 3 cells, typical of 18 examined, are shown.

Fig. 4. Averaged shapes of histamine- and thimerosal-stimulated Ca²⁺ spikes in single HeLa cells.
the episodic discharge of intracellular Ca$^{2+}$ stores. Effects of thimerosal on Ca$^{2+}$ pumping were examined in the result of irreversible damage to intracellular Ca$^{2+}$ stores. The cysteine synthetase inhibitor, DL-buthionine (S,R)-sulfoximine (100 nM) itself was reversible (Fig. 5, a and b). These results, and the observation that thio-  

tic reagents inhibit Ca$^{2+}$-ATPases (Jones et al., 1983; Bellomo et al., 1983; Guillemette and Segui, 1988). The effects of thimerosal on Ca$^{2+}$ pumping were examined in permeabilized cells, in order to study both the uptake into, and efflux from, internal Ca$^{2+}$ stores. Thimerosal caused a decrease in the steady-state Ca$^{2+}$ content of permeabilized cells from $5.6 \pm 0.2$ nmol/mg protein in control cells ($n = 3$) to $3.5 \pm 0.2$ nmol/mg protein ($n = 3$) in cells treated with 100 nM thimerosal (Fig. 7). The unidirectional $^{45}$Ca$^{2+}$ efflux from permeabilized HeLa cells was unaffected by thimerosal (10 nM; Fig. 8), suggesting that its effects on $^{45}$Ca$^{2+}$ accumulation were the sole consequence of thimerosal inhibiting Ca$^{2+}$ uptake. It is noteworthy that 1 nM thimerosal, which sustained Ca$^{2+}$ spiking (Fig. 3) and gave a modest sensitization of InsP$_3$-induced Ca$^{2+}$ release (below), had no effect on the steady-state Ca$^{2+}$ content ($5.8 \pm 0.2$ nmol/mg protein, $n = 3$).

Preincubation of permeabilized cells with thimerosal (5 min) caused a concentration-dependent increase in the sensitivity of the intracellular Ca$^{2+}$ stores to InsP$_3$ (2.6-fold with

Fig. 5. DTT inhibits thimerosal-induced Ca$^{2+}$ spikes. Cells were perfused with Ca$^{2+}$-containing EM supplemented with 100 nM thimerosal for the periods shown. The 3 cells shown in a were briefly exposed (2 min) to 1 mM DTT for the periods shown; DTT rapidly reversed the otherwise irreversible effects of thimerosal. In b, 3 cells were exposed to DTT for a longer period (5.5 min), which was much longer than the normal interspike interval, and again there was complete reversal of the effects of thimerosal. From both panels, the effects of DTT itself are clearly reversible.

Fig. 4. Shapes of histamine- and thimerosal-evoked Ca$^{2+}$ spikes. The figure shows an averaged Ca$^{2+}$ spike, evoked by either 1 nM histamine ($n = 28$) or thimerosal ($n = 66$). ai and bi show the changes in [Ca$^{2+}$];, plotted against time during the spike. aii and bii show [Ca$^{2+}$];, differentiated with respect to time. aiii and biii are phase diagrams displaying the rate of change in [Ca$^{2+}$]; during the spike plotted against [Ca$^{2+}$];. In the phase diagrams, time increases in a clockwise direction and positive $d$[Ca$^{2+}$];/$dt$ indicates an increase in [Ca$^{2+}$];. The data for thimerosal-evoked spikes were taken from experiments such as that shown in Fig. 3 where the basal [Ca$^{2+}$]; did not increase during the experiment.

(DTT) for 2 (Fig. 5a) or 5.5 (Fig. 5b) min; the effect of DTT was itself reversible (Fig. 5, a and b). These results, and the observation that histamine was able to evoke further Ca$^{2+}$ mobilization in cells which had been incubated with thimerosal (Fig. 2b) suggest that the effects of thimerosal are not the result of irreversible damage to intracellular Ca$^{2+}$ stores. Subsequent experiments were designed to address the mechanism underlying the ability of thimerosal to reversibly trigger the episodic discharge of intracellular Ca$^{2+}$ stores.

The interval between the application of thimerosal and the first detectable change in [Ca$^{2+}$]; varied greatly between cells (e.g. Figs. 2a and 5) and could be as long as 15 min. Both this latency and its variability between cells were significantly decreased by preincubating cells for 24 h with the $\gamma$-glutamyl cysteine synthetase inhibitor, DL-buthionine (S,R)-sulfoximine (BSO, 1 mM). The interval between application of thimerosal (100 nM) and the peak of the first Ca$^{2+}$ spike was $4.5 \pm 0.2$ min ($n = 104$) in control cells and $2.4 \pm 0.1$ min ($n = 95$) in cells pretreated with BSO (Fig. 6). The BSO pretreatment, which had no effect on responses to histamine, caused a $72 \pm 1\%$ ($n = 4$) decrease in the intracellular concentration of reduced glutathione (GSH), which is similar to its effects on other cells (Griffith and Meister, 1979; Dethmers and Meister, 1981; Shrieve et al., 1988).

Effect of Thimerosal on Permeabilized Cells — A characteristic feature of the action of thimerosal, particularly at high concentrations, is the gradual elevation of the basal [Ca$^{2+}$]; (Fig. 2a). This is consistent with earlier observations suggesting that thiol reagents inhibit Ca$^{2+}$-ATPases (Jones et al., 1983; Bellomo et al., 1983; Guillemette and Segui, 1988). The effects of thimerosal on Ca$^{2+}$ pumping were examined in permeabilized cells, in order to study both the uptake into, and efflux from, internal Ca$^{2+}$ stores. Thimerosal caused a decrease in the steady-state Ca$^{2+}$ content of permeabilized cells from $5.6 \pm 0.2$ nmol/mg protein in control cells ($n = 3$) to $3.5 \pm 0.2$ nmol/mg protein ($n = 3$) in cells treated with 100 nM thimerosal (Fig. 7). The unidirectional $^{45}$Ca$^{2+}$ efflux from permeabilized HeLa cells was unaffected by thimerosal (10 nM; Fig. 8), suggesting that its effects on $^{45}$Ca$^{2+}$ accumulation were the sole consequence of thimerosal inhibiting Ca$^{2+}$ uptake. It is noteworthy that 1 nM thimerosal, which sustained Ca$^{2+}$ spiking (Fig. 3) and gave a modest sensitization of InsP$_3$-induced Ca$^{2+}$ release (below), had no effect on the steady-state Ca$^{2+}$ content ($5.8 \pm 0.2$ nmol/mg protein, $n = 3$).

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Fig. 6. Sensitization of HeLa cells to thimerosal after depletion of intracellular GSH. HeLa cells were pretreated with BSO (1 mM) for 24 h and then stimulated with thimerosal (100 μM) for the period shown. In cells depleted (by 72%) of intracellular GSH, the Ca²⁺ rise triggered by thimerosal was both more rapid and sustained than in untreated cells (compare with Figs. 2a and 5).

10 μM thimerosal, and a modest decrease in the fraction of the stores released by a maximal InsP₃ concentration (Fig. 9; Table I). This increased sensitivity to InsP₃ occurred despite the decrease in Ca²⁺ content of the intracellular stores, which earlier work suggests may decrease the sensitivity of the InsP₃ receptor to InsP₃ (Nunn and Taylor, 1992). The increased sensitivity of thimerosal-treated cells to InsP₃ was not a consequence of the inhibition of Ca²⁺ uptake because in unidirectional ⁴⁵Ca²⁺ efflux experiments, a submaximal InsP₃ concentration (100 nM) stimulated a greater ⁴⁵Ca²⁺ accumulation by the permeabilized cells.

Table 1

| [InsP₃] (μM) | EC₅₀ for InsP₃ | Maximal release |
|--------------|----------------|-----------------|
| 0            | 161 ± 20       | 77 ± 7          |
| 1            | 105 ± 10       | 70 ± 8          |
| 5            | 82 ± 12        | 63 ± 6          |
| 10           | 62 ± 5         | 61 ± 5          |

Effect of thimerosal on InsP₃-stimulated Ca²⁺ mobilization from permeabilized cells

Permeabilized cells were loaded to steady-state with ⁴⁵Ca²⁺ in the presence or absence of various concentrations of thimerosal (5 min) and the incubations were then continued for a further minute in the presence of a range of concentrations of InsP₃. The results (mean ± S.E. of 3 independent experiments) show that thimerosal decreased the InsP₃ concentration required for half-maximal emptying of the InsP₃-sensitive Ca²⁺ stores (EC₅₀) and slightly reduced the size of the InsP₃-sensitive stores.

Fig. 8. Thimerosal does not affect unidirectional ⁴⁵Ca²⁺ efflux from permeabilized HeLa cells. Permeabilized HeLa cells were loaded to steady-state with ⁴⁵Ca²⁺ and, at t = 0 in the figure, glucose and hexokinase were added to rapidly remove ATP and so prevent further ⁴⁵Ca²⁺ uptake. Control unidirectional ⁴⁵Ca²⁺ efflux (A) is indistinguishable from that occurring when 10 μM thimerosal (C) was added with the glucose and hexokinase. Results (mean ± S.E. of 3 independent experiments) are plotted semi-logarithmically and the lines shown are derived by fitting the data to a mono-exponential equation.

Fig. 9. Thimerosal-induced sensitization of InsP₃-mediated Ca²⁺ release. Permeabilized HeLa cells were loaded to steady-state (5 min) with ⁴⁵Ca²⁺ in the absence (C) or presence (Δ, 5 μM; ○, 10 μM) of thimerosal. The cells were then incubated for a further 60 s with appropriate concentrations of InsP₃. The results (mean ± S.E. of 3 independent experiments) are expressed as fractions of the InsP₃-sensitive Ca²⁺ pool mobilized by each InsP₃ concentration and the curves shown are those derived from fitting the data to a logistic equation (see "Experimental Procedures").

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TABLE I

Effect of thimerosal on Accumulation of Inositol Phosphates—Stimulation of intact HeLa cells for 15 min, in the presence of 30 mM LiCl, with a concentration of histamine (25 μM) that gives a maximal [Ca²⁺]ₗ rise (Bootman et al., 1992), caused a 12-fold increase in total inositol phosphates accumulation (Fig. 10d) reflecting comparable increases in InsP₃ (Fig. 10a), InsP₁ (Fig. 10b), and InsP₂/InsP₄ (Fig. 10c). A lower histamine concentration (1 μM), which evoked lesser Ca²⁺ signals than 100 μM thimerosal, caused a 3-fold increase (Fig. 10). Thimerosal, at a concentration (100 μM) sufficient to evoke substantial increases in [Ca²⁺]ₗ, produced an inhibition of both the basal and histamine-stimulated rates of inositol phosphates formation (Fig. 10). These results suggest
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**Table II**

| Control | 10 nM InsP$_3$ | 4 nM InsP$_3$ |
|---------|----------------|---------------|
| 17 ± 7  | 82 ± 1         |               |
| 45 ± 3  | 83 ± 2         |               |

**Thimerosal Sensitizes the InsP$_3$ Receptor**

Cells were loaded to steady-state (5 min) with or without 10 μM thimerosal. ATP was then rapidly removed by addition of glucose and hexokinase simultaneously with addition of a maximal (4 μM) or submaximal (100 nM) concentration of InsP$_3$ and the incubations were continued for 10 s. The results (mean ± S.E., n = 5 for control and n = 6 for thimerosal-treated cells) show that thimerosal pretreatment increases the sensitivity of the cells to the submaximal InsP$_3$ concentration.

**DISCUSSION**

Histamine, acting at H$_1$ receptors (Sauvé et al., 1987; Bootman et al., 1992), evokes repetitive Ca$^{2+}$ spikes in Fura-2-loaded HeLa cells. This Ca$^{2+}$ spiking occurs in the presence or absence of Ca$^{2+}$o, suggesting that it results from the periodic release of Ca$^{2+}$ from intracellular stores, but the spiking is sustained only in the presence of Ca$^{2+}$o (Fig. 1). Similar changes in [Ca$^{2+}$], have been observed in many cell types after activation of receptors that lead to production of InsP$_3$. Under appropriate conditions, the thiol reagent, thimerosal, evoked repetitive Ca$^{2+}$ spikes in HeLa cells (Fig. 3). The Ca$^{2+}$ spikes evoked by both stimuli were of a similar amplitude and shape. However, the rates of [Ca$^{2+}$], recovery and the thimerosal-evoked spikes were slower than for histamine-evoked spikes (Fig. 4). The slowing of the rate of recovery may have been due to a slight inhibition of Ca$^{2+}$-ATPases, as thimerosal reduced the steady-state Ca$^{2+}$ content of permeabilized HeLa cells (Fig. 7; Table I) without affecting unidirectional Ca$^{2+}$ efflux from preloaded stores (Fig. 8). We conclude that thimerosal caused a concentration-dependent inhibition of Ca$^{2+}$ uptake, and since Ca$^{2+}$ uptake into these stores can also be fully inhibited by the Ca$^{2+}$-ATPase inhibitor thapsigargin (not shown), it seems likely that thimerosal also inhibits a Ca$^{2+}$-ATPase. A similar slowing in the rate of recovery leading to the same degree of spike broadening was observed with t-BHP-evoked spikes in hepatocytes (Rooney et al., 1991). The reason why thimerosal-evoked spikes had a slower rising phase is unclear; such a response could arise from a less synchronous release of intracellular Ca$^{2+}$ stores during thimerosal-evoked Ca$^{2+}$ spiking.

Thimerosal did not mimic the effect of histamine on the accumulation of inositol phosphates, indeed it substantially inhibited both the basal and histamine-stimulated rates of accumulation (Fig. 10). It seems unlikely, therefore, that an increase in InsP$_3$ production can account for the ability of thimerosal to stimulate Ca$^{2+}$ spikes. The stimulation of spiking by thimerosal is likely to result from the alkylation of critical intracellular sulfhydryl groups, because it can be reversed by DTT (Fig. 5), and is more rapid in onset in cells depleted of GSH by prior incubation with BSO (Fig. 6). The potentiation of thimerosal action by BSO presumably reflected the loss of the internal reducing system based on GSH that is responsible for regenerating free sulfhydryl groups.

Thiol reagents are known to modify many proteins involved in Ca$^{2+}$ regulation: they inhibit Ca$^{2+}$-ATPases (Jones et al., 1983; Bellomo et al., 1983; Guillemette and Seguì, 1988); they stimulate the release of Ca$^{2+}$ from sarcoplasmic reticulum (Zaida et al., 1989); they have been shown to sensitize a Ca$^{2+}$-induced Ca$^{2+}$-release mechanism in hamster eggs (Swann, 1991); they modify both ligand binding to, and the activation of, cell surface receptors (Straus, 1984); and they have been shown to decrease the affinity of the InsP$_3$ receptor for InsP$_3$ (Guillemette and Seguì, 1988; Pruijink et al., 1990), decrease the number of InsP$_3$ binding sites (Guillemette and Seguì, 1988), or sensitize InsP$_3$-stimulated Ca$^{2+}$ mobilization (Misson et al., 1991; Renard et al., 1992).

In HeLa cells, the inhibitory effect of thimerosal on Ca$^{2+}$ uptake into intracellular stores and its stimulatory effect on InsP$_3$-induced Ca$^{2+}$ mobilization could both conceivably contribute to the Ca$^{2+}$ spiking observed in intact HeLa cells. Thimerosal-triggered Ca$^{2+}$ spikes are unlikely to result solely from its inhibitory effects on Ca$^{2+}$ uptake into intracellular stores, because the Ca$^{2+}$-ATPase inhibitor, thapsigargin (Jackson et al., 1988; Thastrup et al., 1990), does not evoke repetitive Ca$^{2+}$ spikes in HeLa cells (results not shown). During prolonged incubations with a high thimerosal concentration in the presence of Ca$^{2+}$o, successive spikes progressively broaden and eventually fuse to give a sustained rise in [Ca$^{2+}$]. (Fig. 2a). This possibly results from progressive inhibition of Ca$^{2+}$-ATPases. However, a low concentration of thimerosal (1 μM) in either the presence or absence of Ca$^{2+}$o, and a high concentration (100 μM) in the absence of Ca$^{2+}$o, evoked spikes without a change in the basal [Ca$^{2+}$]. This suggests that thimerosal can evoke spikes under conditions where the cells are able to regulate [Ca$^{2+}$].

It seems unlikely, that pump inhibition can account for Ca$^{2+}$ spiking generation, and the ability of thimerosal to evoke repetitive spikes may therefore result from sensitization of InsP$_3$-induced Ca$^{2+}$ release. Thimerosal increased the sensitivity of intracellular Ca$^{2+}$ stores to InsP$_3$ by up to 2.6-fold (Fig. 9; Table I). This sensitization was also observed in the absence of Ca$^{2+}$-ATPase activity (Table II) indicating that it was a direct effect on the Ca$^{2+}$ release process and not a consequence of inhibiting the opposing action of the Ca$^{2+}$ uptake pathway. A similar sensitization to InsP$_3$ by thimerosal and GSSG has been observed in permeabilized hepatocytes.
Thimerosal Sensitizes the InsP₃ Receptor

(Missiaen et al., 1991; Renard et al., 1992). In mouse oocytes, both InsP₃ and thimerosal trigger Ca²⁺ spiking, but only the former is inhibited by microinjection of heparin (Carroll and Swann, 1992). Our results suggesting that thimerosal increases the sensitivity of the InsP₃ receptor to InsP₃ provide a possible explanation for this observation because if the affinity of the receptor for InsP₃ is increased by thimerosal then the effectiveness of heparin, a competitive antagonist, would be reduced. Alternatively, both InsP₃ and ryanodine receptors may contribute to Ca²⁺ spiking in mouse oocytes and thimerosal may have effects on both receptors (Swann, 1991).

The effects of thimerosal on Ca²⁺ spiking in HeLa cells may be a consequence of its ability to increase the sensitivity of the InsP₃ receptor to InsP₃ and to thereby promote Ca²⁺ mobilization at resting levels of intracellular InsP₃. Although the degree of sensitization is relatively modest (up to 2.6-fold, Table II), similar changes in sensitivity in hepatocytes in response to either cyclic AMP (Burgess et al., 1991; Capiod et al., 1991) or t-BHP (Rooney et al., 1991) generate Ca²⁺ spikes similar to those evoked by activation of receptors that stimulate InsP₃ formation. We conclude that thimerosal evokes repetitive Ca²⁺ spikes in HeLa cells that are similar to those evoked by histamine, it does so without stimulating InsP₃ formation, and may do so by sensitizing the InsP₃ receptor to resting levels of InsP₃.

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