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Oxidation by Thimerosal Increases Calcium Levels in Renal Tubular Cells

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Abstract: The effect of thimerosal, a reactive oxidant, on cytoplasmic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in Madin Darby canine kidney (MDCK) cells was explored by using the Ca^{2+}-sensitive dye fura-2. Thimerosal acted in a concentration-dependent manner with an EC_{50} of 0.5 μM. The Ca^{2+} signal comprised a gradual rise and a sustained elevation. Removal of extracellular Ca^{2+} reduced 80% of the signal. In Ca^{2+}-free medium, the $[Ca^{2+}]_i$ rise induced by 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) was completely inhibited by pretreatment with 5 μM thimerosal. The thimerosal (5 μM)-induced Ca^{2+} release was not changed by inhibition of phospholipase C with 2 μM U73122. Collectively, this study shows that thimerosal induced $[Ca^{2+}]_i$ rises in renal tubular cells via releasing store Ca^{2+} from the endoplasmic reticulum Ca^{2+} stores in a manner independent of phospholipase C activity.

Thimerosal, a sulfhydryl reagent, is used as an antiseptic and preservative. The ability of thimerosal to act on sulfhydryl group is related to the presence of mercury. Thimersal causes a release of Ca^{2+} from intracellular stores in many cells types; this is accompanied by an influx of extracellular calcium, via oxidation of cell membrane proteins and other components (Pintado *et al.* 1995; Chen *et al.* 1998; Tornquist *et al.* 1999; Montero *et al.* 2001; Poirier *et al.* 2001). Oxidation can alter many aspects of cell function, including Ca^{2+} signaling (Sauer *et al.* 2001). Thimerosal may alter the activity of many cell proteins such as ryanodine receptors (Marengo *et al.* 1998; Dulhunty *et al.* 2000), sodium channels (Evans & Bielefeldt 2000), Ca^{2+}-activated K^+ channels (Lang *et al.* 2000), and L-type Ca^{2+} channels (Fearon *et al.* 1999; Greenwood *et al.* 2002). These effects are often related to the ability of thimerosal to release Ca^{2+} or with the sulfhydryl reactivity (Elferink 1999).

A rise in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is a key signal for many pathophysiological processes in cells including necrosis (Berridge *et al.* 1999; Bootman *et al.* 2001). However, an abnormal $[Ca^{2+}]_i$ rise is cytotoxic and can lead to apoptosis, dysfunction of proteins, interference of ion flux, etc. (Clapham 1995). The effect of thimerosal on $[Ca^{2+}]_i$ in renal tubular cells has not been explored. Thimerosal has been shown to cause renal failure in patients (Pfab *et al.* 1996), but the mechanism is unclear. The Madin Darby canine kidney (MDCK) cell line is a useful model for renal research. It has been shown that in this cell, $[Ca^{2+}]_i$ can increase in response to the stimulation of various endogenous and exogenous compounds, such as ATP (Jan *et al.* 1998a), linoleamide (Huang & Jan 2001), estrogens (Jan *et al.* 2001), etc. With fura-2 as a Ca^{2+}-sensitive dye, we have shown that thimerosal induces concentration-dependent $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+} in MDCK cells. The Ca^{2+} responses are characterized, the concentration-response relationships in the presence and absence of extracellular Ca^{2+} are established, and the pathways underlying thimerosal-induced Ca^{2+} influx and Ca^{2+} release are evaluated.

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Materials and Methods

Cell culture. MDCK cells obtained from American Type Culture Collection were cultured in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2/95% air at 37°C.

Solutions. Ca^{2+}-containing medium contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5; pH 7.4. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that Ca^{2+} was substituted with 1 mM EGTA. Drugs were dissolved in water, ethanol or dimethyl sulfoxide. Final concentrations of organic solvents in the experimental solution were less than 0.1% which did not alter basal $[Ca^{2+}]_i$ (n=3; not shown). Thimerosal was dissolved in dimethyl sulfoxide as a 0.1 mM stock and was diluted to the final concentration before assays.

$[Ca^{2+}]_i$ measurements. Trypsinized cells (10^6/ml) were allowed to recover in culture medium for 1 hr before being loaded with 2 μM fura-2/AM for 30 min. at 25° in the same medium. The cells were washed and resuspended in Ca^{2+}-containing medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Kyoto, Japan) by

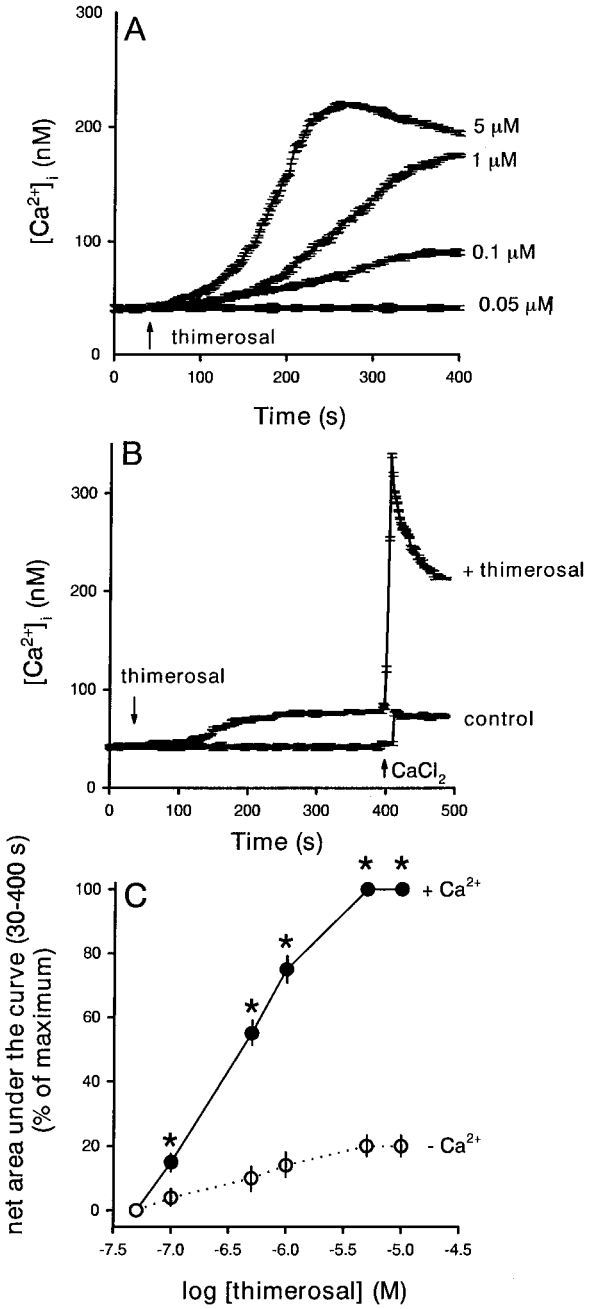

Fig. 1. Effects of thimerosal on $[Ca^{2+}]_i$ in MDCK renal tubular cells. (A) The concentration of thimerosal was 5 μM, 1 μM, 0.1 μM, and 0.05 μM, respectively. Baseline (no thimerosal added) was the same as the trace induced by 0.05 μM thimerosal. The experiments were performed in Ca^{2+}-containing medium. Thimerosal was added at 40 sec. and was present throughout the measurement of 400 sec. (B) Effect of removal of extracellular Ca^{2+} on thimerosal-induced $[Ca^{2+}]_i$ rise and the effect of re-addition of Ca^{2+}. Thimerosal (5 μM) was added at 30 sec. Ca^{2+} (3 mM) was added at 400 ses. Data are means±S.E.M. of 3–5 replicates. Control trace: Ca^{2+} was added without pretreatment with thimerosal. (C) A concentration-response plot of thimerosal-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium and in Ca^{2+}-free medium. The y-axis is the percentage of control. Control is the net (baseline subtracted) integrated area between 30 sec. and 400 sec. (calculated by the Sigmaplot software) of 5 μM thimerosal-induced $[Ca^{2+}]_i$ rise in Ca^{2+}-containing medium. Data are means±S.E.M. of 3–5 replicates. *$P<0.05$ compared with open circles.

recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1 sec. intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously (Jan *et al.* 2001) assuming a K_d of 155 nM (Grynkiewicz *et al.* 1985).

Chemicals. The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). U73122 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione) were from Biomol (Plymouth Meeting, PA, USA). Thimerosal and other reagents were from Sigma (St. Louis, MO, USA).

Statistics. All data are presented as the means±S.E.M. of 3–5 replicates. Statistical comparisons were determined by using ANOVA and a post hoc test, and significance was accepted when $P<0.05$.

Results

Effect of thimerosal on $[Ca^{2+}]_i$.

In the Ca^{2+}-containing medium, basal $[Ca^{2+}]_i$ was 42±2 nM (n=5). Thimerosal (≥0.1 μM) caused an gradual and subsequent $[Ca^{2+}]_i$ rise, which lasted for at least 350 sec. after the addition of thimerosal (fig. 1A); e.g. thimerosal (5 μM)-induced $[Ca^{2+}]_i$ rise attained to 171±3 nM (n=5) over baseline at 230 sec. The signal slowly decayed to 160±2 nM at 400 sec. The effect of thimerosal was concentration-dependent, and saturated at 5 μM of the reagent (figs. 1A and 1C).

Effect of removal of extracellular Ca^{2+} on thimerosal-induced $[Ca^{2+}]_i$ rises.

To examine whether/how influx of extracellular Ca^{2+} and/or mobilization of Ca^{2+} from the intracellular store site(s) may contribute to the thimerosal-induced $[Ca^{2+}]_i$ rises, ef-

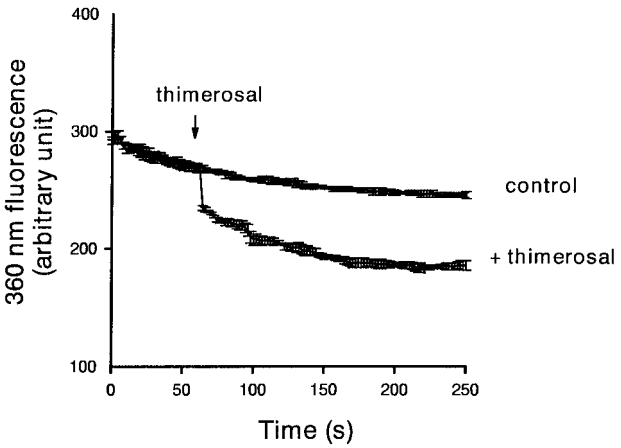

Fig. 2. Effect of thimerosal on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments are performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements started. Y-axis represents the fluorescence changes at the Ca^{2+} -insensitive excitation wavelength (at 360 nm), and the emission wavelength was at 510 nm. Control: fluorescence changes in the absence of thimerosal. Thimerosal (5 μM) was added at 60 sec. Data are means±S.E.M. of three replicates.

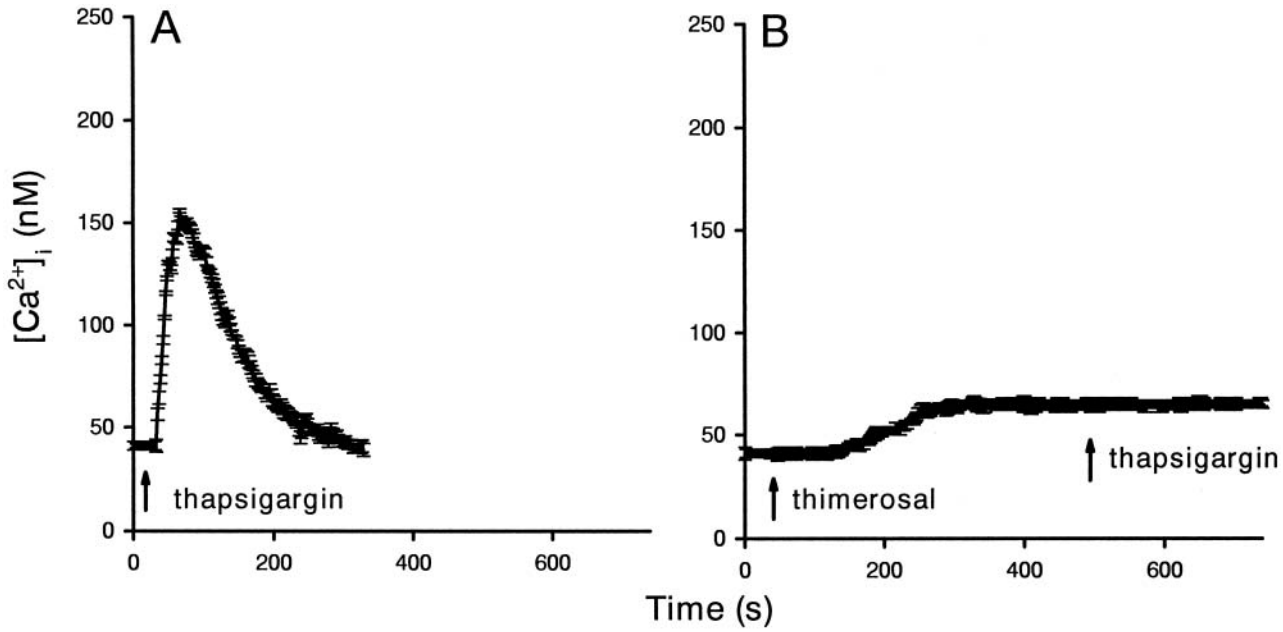

Fig. 3. Intracellular sources of thimerosal-induced $[Ca^{2+}]_i$ rise. The experiments were performed in Ca^{2+}-free medium. (A) Thapsigargin (1 μM) was added at 30 sec. (B) Thimerosal (5 μM) was added at 30 sec. followed by 1 μM thapsigargin added at 500 sec. Data are means±S.E.M. of 3–5 replicates.

fect of thimerosal on $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+} (fig. 1B). The $[Ca^{2+}]_i$ rise caused by 5 μM thimerosal was attenuated, with no change in basal $[Ca^{2+}]_i$ (42±1 nM, n=5). Thimerosal (5 μM) increased $[Ca^{2+}]_i$ by 30±1 nM over baseline. Fig. 1C shows that removal of extracellular Ca^{2+} inhibited thimerosal (0.1–10 μM)-induced $[Ca^{2+}]_i$ rises by about 80%. Fig. 1B also shows that after 5 μM thimerosal pretreatment, addition of Ca^{2+} (3 mM) at 400 sec. induced an immediate $[Ca^{2+}]_i$ rise with a maximum value of 229±2 nM, which was 9 times greater

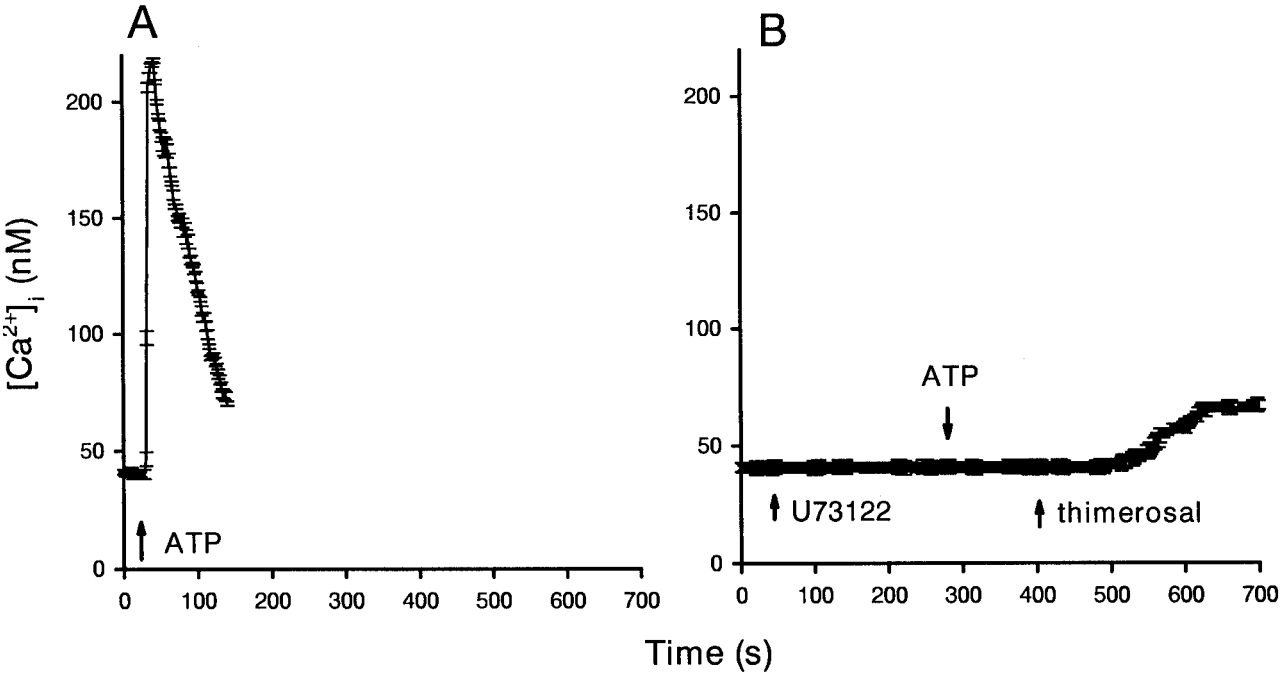

Fig. 4. Effect of U73122 on thimerosal-induced $[Ca^{2+}]_i$ rise. All experiments were performed in Ca^{2+}-freemedium. (A) ATP (10 μM) was added at 30 sec. (B) U73122 (2 μM), ATP (10 μM), and thimerosal (5 μM) were added at 40, 290 and 400 sec., respectively. Data are means±S.E.M. of 3–5 replicates.

than control (25±2 nM; without thimerosal pretreatment; n=4; P<0.05). This suggests that thimerosal opened cell surface Ca^{2+} channels.

Effect of thimerosal on Mn^{2+}-induced quench of fura-2 fluorescence.

These experiments were performed to confirm that the reduced thimerosal-induced $[Ca^{2+}]_i$ rises by removal of extracellular Ca^{2+}, was not due to EGTA-induced store Ca^{2+} depletion. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (Merritt *et al.* 1989). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm (emission wavelength at 510 nm) by Mn^{2+} indicates Ca^{2+} influx. Fig. 2 shows that 5 μM thimerosal induced a gradual decrease in the 360 nm excitation signal by 81±2 (n=5; P<0.05) arbitrary units below control at 170 sec., and the decrease sustained for at least 80 sec.

Mobilization by thimerosal of intracellular Ca^{2+} from the endoplasmic reticulum.

We examined whether thimerosal-induced $[Ca^{2+}]_i$ rises may involve the mobilization of intracellular Ca^{2+} sequestered within the endoplasmic reticulum, a major Ca^{2+} store in MDCK cells (Jan *et al.* 1985a, b). Fig. 3A shows that in Ca^{2+}-free medium, 1 μM thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al.* 1990), increased $[Ca^{2+}]_i$ by 109±2 nM (n=4) in a monophasic manner, followed by a slow decay that returned to baseline within 300 sec. after addition of the reagent. However, fig. 3B shows that after 5 μM thimerosal released store Ca^{2+} for 8 min., thapsigargin (1 μM) failed to induce a $[Ca^{2+}]_i$ rise (n=4; P<0.05).

Lack of involvement of phospholipase C in thimerosal-induced Ca^{2+} release.

The role of phospholipase C-inositol 1,4,5-trisphosphate (IP_3) pathway in thimerosal-induced intracellular Ca^{2+} mobilization from the endoplasmic reticulum was investigated. Fig. 4A shows that 10 μM ATP, an agonist for P2Y type ATP receptors that mobilizes intracellular Ca^{2+} from the endoplasmic reticulum via activation of phospholipase C (Jan *et al.* 1998a), caused an instantaneous monophasic $[Ca^{2+}]_i$ rise (171±2 nM, n=4) in Ca^{2+}-free medium. Fig. 4B, however, shows that pretreatment with 2 μM U73122, an inhibitor of phospholipase C (Thompson *et al.* 1991), abolished ATP-induced $[Ca^{2+}]_i$ rise; in contrast, 10 μM U73343, a biologically inactive analogue of U73122 (Thompson *et al.* 1991), failed to prevent ATP-induced $[Ca^{2+}]_i$ rise (data not shown; n=3). Even in the presence of 2 μM U73122, 5 μM thimerosal caused a significant $[Ca^{2+}]_i$ rise by 31±2 nM (n=4), which was indistinguishable from the control thimerosal response (fig. 1B).

Discussion

The present study has examined the effect of the oxidant thimerosal on $[Ca^{2+}]_i$ in MDCK renal tubular cells. Thimerosal was found to cause a concentration-dependent $[Ca^{2+}]_i$ rise. This represents the first evidence that thimerosal may modulate renal function. An increase in $[Ca^{2+}]_i$ has been shown to be a key message for normal renal function. The balance of a high extracellular osmolarity in the kidney medulla is regulated by osmolytes in the cells. The control of cell volume during hypotonic conditions results in a Ca^{2+}-dependent release of osmolytes (Tinel *et al.* 2000). Many endogenous compounds, such as ATP and bradykinin, activate renal cells via causing a well-tuned $[Ca^{2+}]_i$ rise (Jan *et al.* 1998a & b). However, an uncontrolled $[Ca^{2+}]_i$ rise may lead to tubular injury resulting in a profound fall in glomerular filtration rate, including increased tubuloglomerular feedback and distal tubular obstruction, in ischemic acute renal failure (Edelstein *et al.* 1997). In light of the report that acute poisoning by thimerosal in human can lead to renal failure (Pfab *et al.* 1996) and the fact that thimerosal is used as a preservative in many vaccine preparations (Elferink 1999), the *in vivo* renal effect of thimerosal needs to be studied.

The data show that thimerosal increases $[Ca^{2+}]_i$ by causing both store Ca^{2+} release and extracellular Ca^{2+} influx because the response was reduced by 80% by removal of extracellular Ca^{2+}. The thimerosal-induced Ca^{2+} influx was independently confirmed by thimerosal-induced Mn^{2+} quench of fura-2 fluorescence at the Ca^{2+}-insensitive 360 nm excitation wavelength. Thimerosal may cause Ca^{2+} influx via store-operated Ca^{2+} entry, a process triggered by store Ca^{2+} depletion (Putney 1986) that has been previously shown to play a main role in Ca^{2+} influx in MDCK cells (Huang & Jan 2001; Jan *et al.* 2001).

Regarding the intracellular Ca^{2+} stores of the thimerosal response, the thapsigargin-sensitive endoplasmic reticulum store, the dominant Ca^{2+} store in MDCK cells (Jan *et al.* 2001), appears to play a major role because thimerosal completely depleted the endoplasmic reticulum Ca^{2+} store. The thimerosal-induced Ca^{2+} release does not require a preceding elevation in cytosolic IP_3 levels because this release was unaltered by suppression of phospholipase C activity. The IP_3-independent component(s) of the Ca^{2+} releasing event is unknown, but may be related to inhibition of Ca^{2+} pump or permeabilization of the endoplasmic reticulum membranes. Although thimerosal has been shown to modulate ryanodine channels in excitable cells (Marengo *et al.* 1998); however, MDCK cells do not possess active ryanodine-sensitive Ca^{2+} stores as demonstrated previously (Jan *et al.* 1998b). Together, the present study shows that thimerosal induces a concentration-dependent, sustained $[Ca^{2+}]_i$ rise in renal tubular cells via releasing store Ca^{2+} and causing Ca^{2+} influx.

Acknowledgements

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