

## 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  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in Madin Darby canine kidney (MDCK) cells was explored by using the  $\text{Ca}^{2+}$ -sensitive dye fura-2. Thimerosal acted in a concentration-dependent manner with an  $\text{EC}_{50}$  of 0.5  $\mu\text{M}$ . The  $\text{Ca}^{2+}$  signal comprised a gradual rise and a sustained elevation. Removal of extracellular  $\text{Ca}^{2+}$  reduced 80% of the signal. In  $\text{Ca}^{2+}$ -free medium, the  $[\text{Ca}^{2+}]_i$  rise induced by 1  $\mu\text{M}$  thapsigargin (an endoplasmic reticulum  $\text{Ca}^{2+}$  pump inhibitor) was completely inhibited by pretreatment with 5  $\mu\text{M}$  thimerosal. The thimerosal (5  $\mu\text{M}$ )-induced  $\text{Ca}^{2+}$  release was not changed by inhibition of phospholipase C with 2  $\mu\text{M}$  U73122. Collectively, this study shows that thimerosal induced  $[\text{Ca}^{2+}]_i$  rises in renal tubular cells via releasing store  $\text{Ca}^{2+}$  from the endoplasmic reticulum  $\text{Ca}^{2+}$  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. Thimerosal causes a release of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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),  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Lang *et al.* 2000), and L-type  $\text{Ca}^{2+}$  channels (Fearon *et al.* 1999; Greenwood *et al.* 2002). These effects are often related to the ability of thimerosal to release  $\text{Ca}^{2+}$  or with the sulfhydryl reactivity (Elferink 1999).

A rise in intracellular free  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) is a key signal for many pathophysiological processes in cells including necrosis (Berridge *et al.* 1999; Bootman *et al.* 2001). However, an abnormal  $[\text{Ca}^{2+}]_i$  rise is cytotoxic and can lead to apoptosis, dysfunction of proteins, interference of ion flux, etc. (Clapham 1995). The effect of thimerosal on  $[\text{Ca}^{2+}]_i$  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,  $[\text{Ca}^{2+}]_i$  can increase in response to the stimulation of various en-

dogenous 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  $\text{Ca}^{2+}$ -sensitive dye, we have shown that thimerosal induces concentration-dependent  $[\text{Ca}^{2+}]_i$  rises both in the presence and absence of extracellular  $\text{Ca}^{2+}$  in MDCK cells. The  $\text{Ca}^{2+}$  responses are characterized, the concentration-response relationships in the presence and absence of extracellular  $\text{Ca}^{2+}$  are established, and the pathways underlying thimerosal-induced  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  release are evaluated.

### 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%  $\text{CO}_2/95\%$  air at 37°C.

**Solutions.**  $\text{Ca}^{2+}$ -containing medium contained (in mM): NaCl 140; KCl 5;  $\text{MgCl}_2$  1;  $\text{CaCl}_2$  2; Hepes 10; glucose 5; pH 7.4.  $\text{Ca}^{2+}$ -free medium contained similar components as  $\text{Ca}^{2+}$ -containing medium except that  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$  ( $n=3$ ; not shown). Thimerosal was dissolved in dimethyl sulfoxide as a 0.1 mM stock and was diluted to the final concentration before assays.

**$[\text{Ca}^{2+}]_i$  measurements.** Trypsinized cells ( $10^6/\text{ml}$ ) were allowed to recover in culture medium for 1 hr before being loaded with 2  $\mu\text{M}$  fura-2/AM for 30 min. at 25° in the same medium. The cells were washed and resuspended in  $\text{Ca}^{2+}$ -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

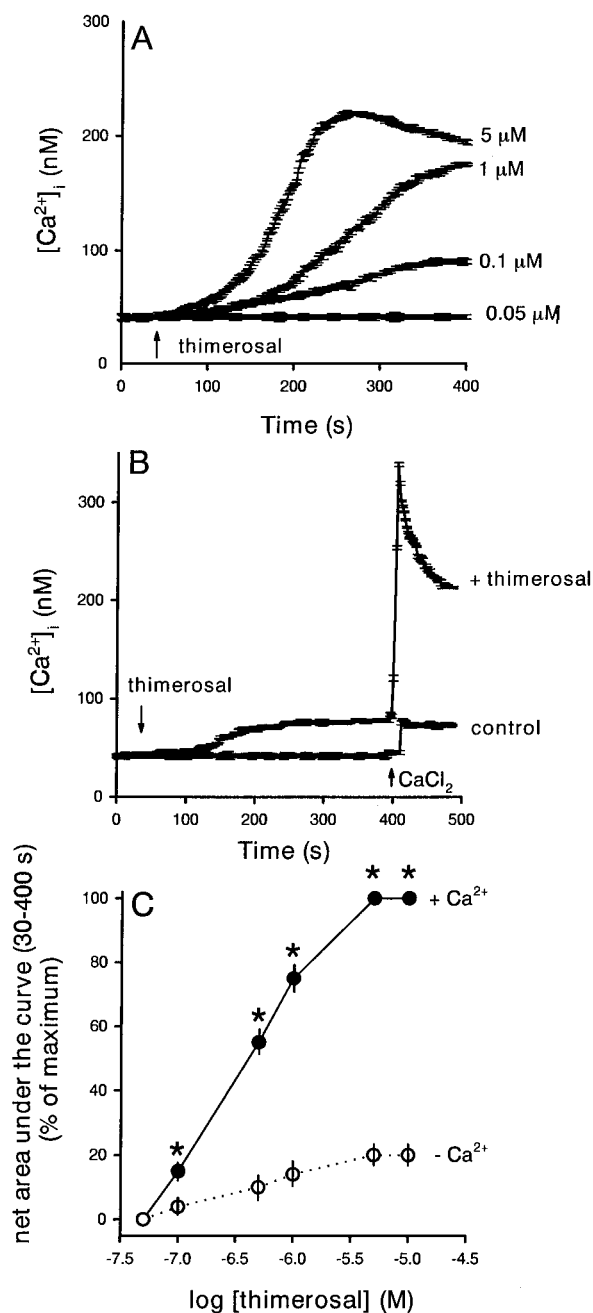


Fig. 1. Effects of thimerosal on  $[Ca^{2+}]_i$  in MDCK renal tubular cells. (A) The concentration of thimerosal was 5  $\mu$ M, 1  $\mu$ M, 0.1  $\mu$ M, and 0.05  $\mu$ M, respectively. Baseline (no thimerosal added) was the same as the trace induced by 0.05  $\mu$ M thimerosal. The experiments were 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  $\mu$ M) was added at 30 sec.  $Ca^{2+}$  (3 mM) was added at 400 sec. Data are means  $\pm$  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  $\mu$ M thimerosal-induced  $[Ca^{2+}]_i$  rise in  $Ca^{2+}$ -containing medium. Data are means  $\pm$  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-pyrrolidinedione) 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  $\pm$  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  $42 \pm 2$  nM ( $n = 5$ ). Thimerosal ( $\geq 0.1$   $\mu$ M) 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  $\mu$ M)-induced  $[Ca^{2+}]_i$  rise attained to  $171 \pm 3$  nM ( $n = 5$ ) over baseline at 230 sec. The signal slowly decayed to  $160 \pm 2$  nM at 400 sec. The effect of thimerosal was concentration-dependent, and saturated at 5  $\mu$ M 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-

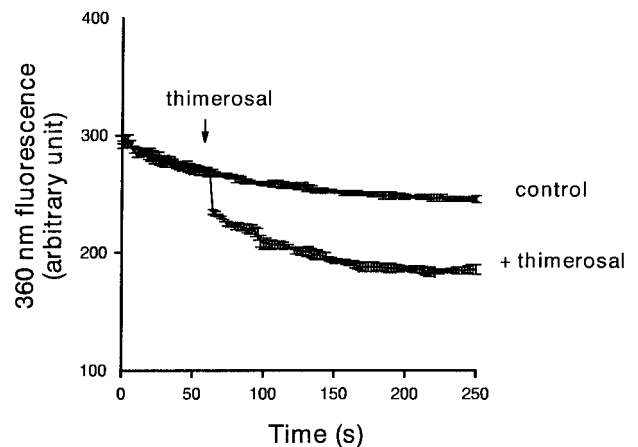


Fig. 2. Effect of thimerosal on  $Ca^{2+}$  influx by measuring  $Mn^{2+}$  quench of fura-2 fluorescence. Experiments are performed in  $Ca^{2+}$ -containing medium.  $MnCl_2$  (50  $\mu$ M) was added to cells before fluorescence measurements started. Y-axis represents the fluorescence changes at the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

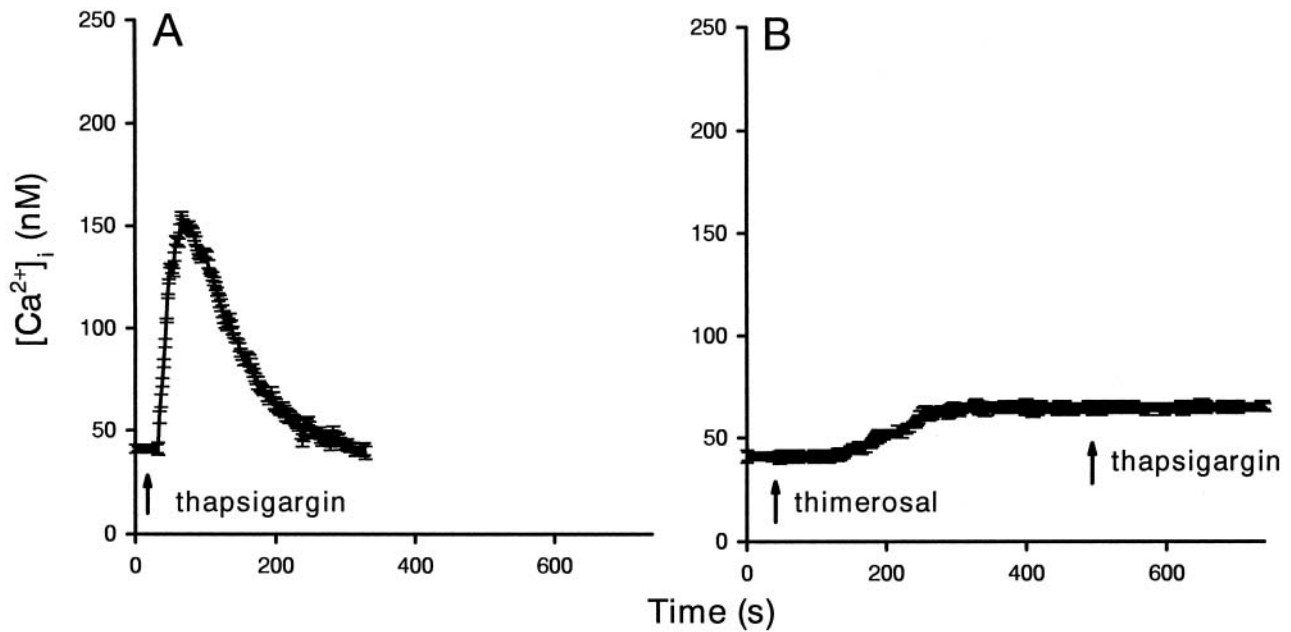


Fig. 3. Intracellular sources of thimerosal-induced  $[Ca^{2+}]_i$  rise. The experiments were performed in  $Ca^{2+}$ -free medium. (A) Thapsigargin (1  $\mu$ M) was added at 30 sec. (B) Thimerosal (5  $\mu$ M) was added at 30 sec. followed by 1  $\mu$ M thapsigargin added at 500 sec. Data are means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

effect 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  $\mu$ M thimerosal was attenuated, with no change in basal  $[Ca^{2+}]_i$  ( $42 \pm 1$  nM,  $n=5$ ). Thimerosal (5  $\mu$ M) increased  $[Ca^{2+}]_i$  by  $30 \pm 1$  nM over baseline. Fig. 1C shows that re-

moval of extracellular  $Ca^{2+}$  inhibited thimerosal (0.1–10  $\mu$ M)-induced  $[Ca^{2+}]_i$  rises by about 80%. Fig. 1B also shows that after 5  $\mu$ M thimerosal pretreatment, addition of  $Ca^{2+}$  (3 mM) at 400 sec. induced an immediate  $[Ca^{2+}]_i$  rise with a maximum value of  $229 \pm 2$  nM, which was 9 times greater

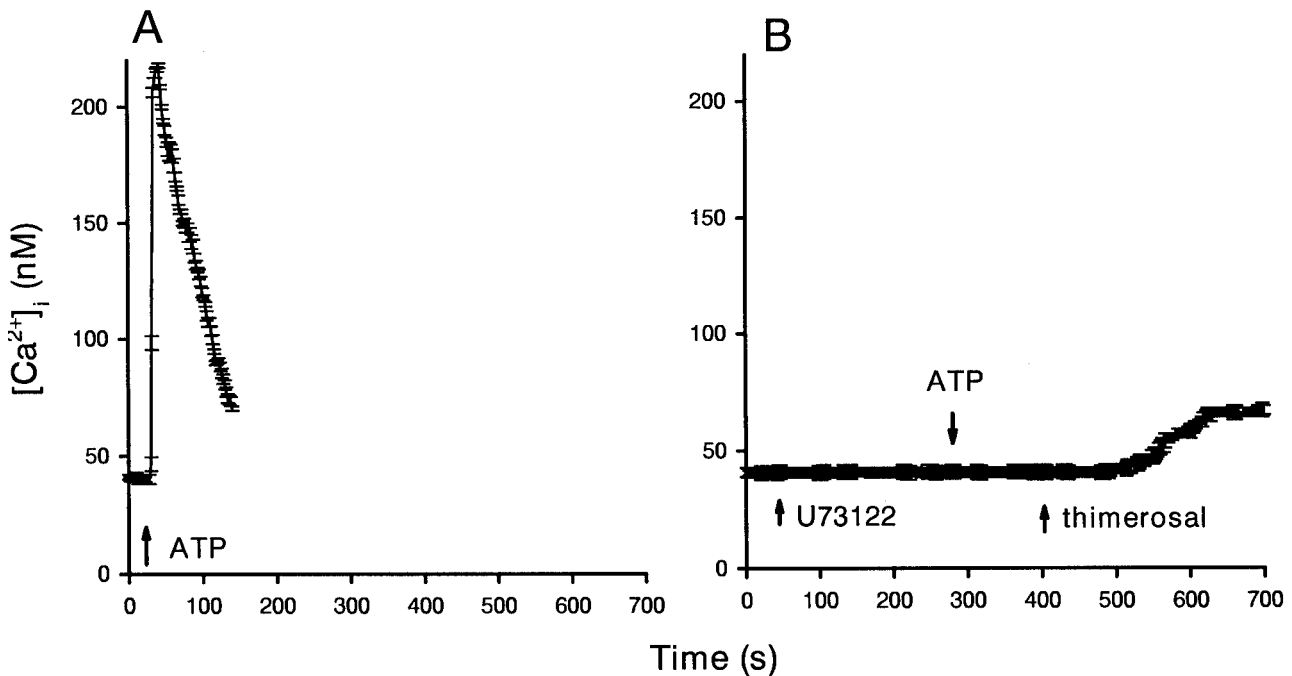


Fig. 4. Effect of U73122 on thimerosal-induced  $[Ca^{2+}]_i$  rise. All experiments were performed in  $Ca^{2+}$ -free medium. (A) ATP (10  $\mu$ M) was added at 30 sec. (B) U73122 (2  $\mu$ M), ATP (10  $\mu$ M), and thimerosal (5  $\mu$ M) were added at 40, 290 and 400 sec., respectively. Data are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

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

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

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

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

We examined whether thimerosal-induced  $[\text{Ca}^{2+}]_i$  rises may involve the mobilization of intracellular  $\text{Ca}^{2+}$  sequestered within the endoplasmic reticulum, a major  $\text{Ca}^{2+}$  store in MDCK cells (Jan *et al.* 1985a, b). Fig. 3A shows that in  $\text{Ca}^{2+}$ -free medium, 1  $\mu\text{M}$  thapsigargin, an inhibitor of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (Thastrup *et al.* 1990), increased  $[\text{Ca}^{2+}]_i$  by  $109 \pm 2$  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  $\mu\text{M}$  thimerosal released store  $\text{Ca}^{2+}$  for 8 min., thapsigargin (1  $\mu\text{M}$ ) failed to induce a  $[\text{Ca}^{2+}]_i$  rise ( $n=4$ ;  $P<0.05$ ).

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

The role of phospholipase C-inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) pathway in thimerosal-induced intracellular  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum was investigated. Fig. 4A shows that 10  $\mu\text{M}$  ATP, an agonist for  $\text{P}_2\text{Y}$  type ATP receptors that mobilizes intracellular  $\text{Ca}^{2+}$  from the endoplasmic reticulum via activation of phospholipase C (Jan *et al.* 1998a), caused an instantaneous monophasic  $[\text{Ca}^{2+}]_i$  rise ( $171 \pm 2$  nM,  $n=4$ ) in  $\text{Ca}^{2+}$ -free medium. Fig. 4B, however, shows that pretreatment with 2  $\mu\text{M}$  U73122, an inhibitor of phospholipase C (Thompson *et al.* 1991), abolished ATP-induced  $[\text{Ca}^{2+}]_i$  rise; in contrast, 10  $\mu\text{M}$  U73343, a biologically inactive analogue of U73122 (Thompson *et al.* 1991), failed to prevent ATP-induced  $[\text{Ca}^{2+}]_i$  rise (data not shown;  $n=3$ ). Even in the presence of 2  $\mu\text{M}$  U73122, 5  $\mu\text{M}$  thimerosal caused a significant  $[\text{Ca}^{2+}]_i$  rise by  $31 \pm 2$  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  $[\text{Ca}^{2+}]_i$  in MDCK renal tubular cells. Thi-

merosal was found to cause a concentration-dependent  $[\text{Ca}^{2+}]_i$  rise. This represents the first evidence that thimerosal may modulate renal function. An increase in  $[\text{Ca}^{2+}]_i$  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  $\text{Ca}^{2+}$ -dependent release of osmolytes (Tinel *et al.* 2000). Many endogenous compounds, such as ATP and bradykinin, activate renal cells via causing a well-tuned  $[\text{Ca}^{2+}]_i$  rise (Jan *et al.* 1998a & b). However, an uncontrolled  $[\text{Ca}^{2+}]_i$  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 (Pfaff *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  $[\text{Ca}^{2+}]_i$  by causing both store  $\text{Ca}^{2+}$  release and extracellular  $\text{Ca}^{2+}$  influx because the response was reduced by 80% by removal of extracellular  $\text{Ca}^{2+}$ . The thimerosal-induced  $\text{Ca}^{2+}$  influx was independently confirmed by thimerosal-induced  $\text{Mn}^{2+}$  quench of fura-2 fluorescence at the  $\text{Ca}^{2+}$ -insensitive 360 nm excitation wavelength. Thimerosal may cause  $\text{Ca}^{2+}$  influx via store-operated  $\text{Ca}^{2+}$  entry, a process triggered by store  $\text{Ca}^{2+}$  depletion (Putney 1986) that has been previously shown to play a main role in  $\text{Ca}^{2+}$  influx in MDCK cells (Huang & Jan 2001; Jan *et al.* 2001).

Regarding the intracellular  $\text{Ca}^{2+}$  stores of the thimerosal response, the thapsigargin-sensitive endoplasmic reticulum store, the dominant  $\text{Ca}^{2+}$  store in MDCK cells (Jan *et al.* 2001), appears to play a major role because thimerosal completely depleted the endoplasmic reticulum  $\text{Ca}^{2+}$  store. The thimerosal-induced  $\text{Ca}^{2+}$  release does not require a preceding elevation in cytosolic  $\text{IP}_3$  levels because this release was unaltered by suppression of phospholipase C activity. The  $\text{IP}_3$ -independent component(s) of the  $\text{Ca}^{2+}$  releasing event is unknown, but may be related to inhibition of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  stores as demonstrated previously (Jan *et al.* 1998b). Together, the present study shows that thimerosal induces a concentration-dependent, sustained  $[\text{Ca}^{2+}]_i$  rise in renal tubular cells via releasing store  $\text{Ca}^{2+}$  and causing  $\text{Ca}^{2+}$  influx.

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