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 Ca2+ concentrations ([Ca2+]i) in Madin Darby canine kidney (MDCK) cells was explored by using the Ca2+-sensitive dye fura-2. Thimerosal acted in a concentration-dependent manner with an EC50 of 0.5 μM. The Ca2+ signal comprised a gradual rise and a sustained elevation. Removal of extracellular Ca2+ reduced 80% of the signal. In Ca2+-free medium, the [Ca2+]i rise induced by 1 μM thapsigargin (an endoplasmic reticulum Ca2+ pump inhibitor) was completely inhibited by pretreatment with 5 μM thimerosal. The thimerosal (5 μM)-induced Ca2+ release was not changed by inhibition of phospholipase C with 2 μM U73122. Collectively, this study shows that thimerosal induced [Ca2+]i rises in renal tubular cells via releasing store Ca2+ from the endoplasmic reticulum Ca2+ stores in a manner independent of phospholipase C activity.

Thimerosal, a sulphydryl reagent, is used as an antiseptic and preservative. The ability of thimerosal to act on sulphydryl group is related to the presence of mercury. Thimerosal causes a release of Ca2+ from intracellular stores in many cells types; this is accompanied by an influx of extracellular Ca2+ (Pintado et al. 1995; Chen et al. 1998; Torququist et al. 1999; Montero et al. 2001; Poirier et al. 2001). Oxidation can alter many aspects of cell function, including Ca2+ 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), Ca2+-activated K+ channels (Lang et al. 2000), and L-type Ca2+ channels (Fearon et al. 1999; Greenwood et al. 2002). These effects are often related to the ability of thimerosal to release Ca2+ or with the sulphydryl reactivity (Ellerink 1999).

A rise in intracellular free Ca2+ concentrations ([Ca2+]i) is a key signal for many pathophysiological processes in cells including necrosis (Berridge et al. 1999; Bootman et al. 2001). However, an abnormal [Ca2+]i rise is cytotoxic and can lead to apoptosis, dysfunction of proteins, interference of ion flux, etc. (Clapham 1995). The effect of thimerosal on [Ca2+]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, [Ca2+]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 Ca2+-sensitive dye, we have shown that thimerosal induces concentration-dependent [Ca2+]i rises both in the presence and absence of extracellular Ca2+ in MDCK cells. The Ca2+ responses are characterized by the concentration-response relationships in the presence and absence of extracellular Ca2+ are established, and the pathways underlying thimerosal-induced Ca2+ influx and Ca2+ 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% CO2/95% air at 37°C.

Solutions. Ca2+-containing medium contained (in mM): NaCl 140; KCl 5; MgCl2 1; CaCl2 2; Hepes 10; glucose 5; pH 7.4. Ca2+-free medium contained similar components as Ca2+-containing medium except that CaCl2 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 [Ca2+]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.

[Ca2+]i measurements. Trypsinized cells (106/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°C in the same medium. The cells were washed and resuspended in Ca2+-containing medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) 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 \([\text{Ca}^{2+}]_i\) in MDCK renal tubular cells. (A) The concentration of thimerosal was 5 \(\mu\text{M}\), 1 \(\mu\text{M}\), 0.1 \(\mu\text{M}\), and 0.05 \(\mu\text{M}\), respectively. Baseline (no thimerosal added) was the same as the trace induced by 0.05 \(\mu\text{M}\) thimerosal. The experiments were performed in \(\text{Ca}^{2+}\)-containing medium. Thimerosal was added at 40 sec. and was present throughout the measurement of 400 sec. (B) Effect of removal of extracellular \(\text{Ca}^{2+}\) on thimerosal-induced \([\text{Ca}^{2+}]_i\) rise and the effect of re-addition of \(\text{Ca}^{2+}\). Thimerosal (5 \(\mu\text{M}\)) was added at 30 sec. \(\text{Ca}^{2+}\) (3 mM) was added at 400 sec. Data are means \(\pm\) S.E.M. of 3–5 replicates. Control trace: \(\text{Ca}^{2+}\) was added without pretreatment with thimerosal. (C) A concentration-response plot of thimerosal-induced \([\text{Ca}^{2+}]_i\) rises in \(\text{Ca}^{2+}\)-containing medium and in \(\text{Ca}^{2+}\)-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 \(\mu\text{M}\) thimerosal-induced \([\text{Ca}^{2+}]_i\) rise in \(\text{Ca}^{2+}\)-containing medium. Data are means \(\pm\) S.E.M. of 3–5 replicates. *\(P<0.05\) compared with open circles.

Fig. 2. Effect of thimerosal on \([\text{Ca}^{2+}]_i\) by measuring Mn\(^2+\)-quench of fura-2 fluorescence. Experiments are performed in \(\text{Ca}^{2+}\)-containing medium. MnCl\(_2\) (50 \(\mu\text{M}\)) was added to cells before fluorescence measurements started. Y-axis represents the fluorescence changes at the \(\text{Ca}^{2+}\)-insensitive excitation wavelength (at 360 nm), and the emission wavelength was at 510 nm. Control: fluorescence changes in the absence of thimerosal. Thimerosal (5 \(\mu\text{M}\)) was added at 60 sec. Data are means \(\pm\) S.E.M. of three replicates.
Fig. 3. Intracellular sources of thimerosal-induced \([\text{Ca}^{2+}]_i\) 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±S.E.M. of 3–5 replicates.

Fig. 4. Effect of U73122 on thimerosal-induced \([\text{Ca}^{2+}]_i\) 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±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 Ca²⁺ channels.

**Effect of thimerosal on Mn²⁺-induced quench of fura-2 fluorescence.**

These experiments were performed to confirm that the reduced thimerosal-induced [Ca²⁺]ᵢ rises by removal of extracellular Ca²⁺, as was not due to EGTA-induced store Ca²⁺ depletion. Mn²⁺ enters cells through similar pathways as Ca²⁺ but quenches fura-2 fluorescence at all excitation wavelengths (Merritt et al. 1989). Thus, quench of fura-2 fluorescence excited at the Ca²⁺-insensitive excitation wavelength of 360 nm (emission wavelength of 510 nm) by Mn²⁺ indicates Ca²⁺ 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 Ca²⁺ from the endoplasmic reticulum.**

We examined whether thimerosal-induced [Ca²⁺]ᵢ rises may involve the mobilization of intracellular Ca²⁺ sequestered within the endoplasmic reticulum, a major Ca²⁺ store in MDCK cells (Jan et al. 1985a, b). Fig. 3A shows that in Ca²⁺-free medium, 1 μM thapsigargin, an inhibitor of endoplasmic reticulum Ca²⁺-ATPase (Thastrup et al. 1990), increased [Ca²⁺]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 Ca²⁺ for 8 min., thapsigargin (1 μM) failed to induce a [Ca²⁺]ᵢ rise (n=4; P<0.05).

**Lack of involvement of phospholipase C in thimerosal-induced Ca²⁺ release.**

The role of phospholipase C-inositol 1,4,5-trisphosphate (IP₃) pathway in thimerosal-induced intracellular Ca²⁺ mobilization from the endoplasmic reticulum was investigated. Fig. 4A shows that 10 μM ATP, an agonist for P2Y type ATP receptors that mobilizes intracellular Ca²⁺ from the endoplasmic reticulum via activation of phospholipase C (Jan et al. 1998a), caused an instantaneous monophasic [Ca²⁺]ᵢ rise (171±2 nM, n=4) in Ca²⁺-free medium. Fig. 4B, however, shows that pretreatment with 2 μM U73122, an inhibitor of phospholipase C (Thompson et al. 1991), abolished ATP-induced [Ca²⁺]ᵢ rise; in contrast, 10 μM U73343, a biologically inactive analogue of U73122 (Thompson et al. 1991), failed to prevent ATP-induced [Ca²⁺]ᵢ rise (data not shown; n=3). Even in the presence of 2 μM U73122, 5 μM thimerosal caused a significant [Ca²⁺]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 [Ca²⁺]ᵢ in MDCK renal tubular cells. Thimerosal was found to cause a concentration-dependent [Ca²⁺]ᵢ rise. This represents the first evidence that thimerosal may modulate renal function. An increase in [Ca²⁺]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 Ca²⁺-dependent release of osmolytes (Tinel et al. 2000). Many endogenous compounds, such as ATP and bradykinin, activate renal cells via causing a well-tuned [Ca²⁺]ᵢ rise (Jan et al. 1998a & b). However, an uncontrolled [Ca²⁺]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 [Ca²⁺]ᵢ, by causing both store Ca²⁺ release and extracellular Ca²⁺ influx because the response was reduced by 80% by removal of extracellular Ca²⁺. The thimerosal-induced Ca²⁺ influx was independently confirmed by thimerosal-induced Mn²⁺ quench of fura-2 fluorescence at the Ca²⁺-insensitive 360 nm excitation wavelength. Thimerosal may cause Ca²⁺ influx via store-operated Ca²⁺ entry, a process triggered by store Ca²⁺ depletion (Putney 1986) that has been previously shown to play a main role in Ca²⁺ influx in MDCK cells (Huang & Jan 2001; Jan et al. 2001).

Regarding the intracellular Ca²⁺ stores of the thimerosal response, the thapsigargin-sensitive endoplasmic reticulum store, the dominant Ca²⁺ store in MDCK cells (Jan et al. 2001), appears to play a major role because thimerosal completely depleted the endoplasmic reticulum Ca²⁺ store. The thimerosal-induced Ca²⁺ release does not require a preceding elevation in cytosolic IP₃ levels because this release was unaltered by suppression of phospholipase C activity. The IP₃-independent component(s) of the Ca²⁺ releasing event is unknown, but may be related to inhibition of Ca²⁺ 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 Ca²⁺ stores as demonstrated previously (Jan et al. 1998b). Together, the present study shows that thimerosal induces a concentration-dependent, sustained [Ca²⁺]ᵢ rise in renal tubular cells via releasing store Ca²⁺ and causing Ca²⁺ influx.

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