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THE EFFECTS OF THIMEROSAL, A SULFHYDRYL REAGENT, ON PHASIC MYOMETRIAL CONTRACTIONS

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Thimerosal inhibits calcium uptake and IP_3-induced calcium release from IP_3-sensitive endoplasmic reticulum; this study sought to evaluate the effects of thimerosal on agonist-stimulated phasic myometrial contractions. Thimerosal was found to significantly inhibit phasic contractions stimulated by oxytocin, aluminum fluoride, potassium chloride, ionomycin, and Bay K 8644. These observations provide support for the hypothesis that calcium uptake and IP_3-induced calcium release are important events during agonist-stimulated phasic myometrial contractions.

Cytosolic calcium oscillations in various nonexcitable cells have been associated with cycles of calcium release and refill of inositol 1,4,5-trisphosphate (IP3)-sensitive and insensitive endoplasmic reticulum calcium stores. Release of calcium from the IP_3-sensitive calcium stores is dependent upon opening of the IP_3 receptor/channel (IP_3R). Thimerosal, a sulphydryl reagent, has previously been reported to modulate the activity of the IP_3R in both permeabilized and intact cells [1-4]. Utilizing a monoclonal antibody specific for the IP_3R, Miyazaki et al. [5] demonstrated that the thimerosal effects require direct interaction with the IP_3R protein. These effects of thimerosal have been shown to be dose related; ie. at low concentrations (1-10 μM) thimerosal stimulates cytosolic calcium oscillations, whereas at higher concentrations it inhibits calcium oscillations, produces a sustained increase in cytosolic calcium and/or inhibits IP_3-induced calcium release [1,4,6]. Previous reports have also suggested that thimerosal inhibits the endoplasmic reticulum calcium ATPase pump [2,6]. The thimerosal effects have also been found to be reversed by dithiothreitol (DTT) (a thiol reducing agent) [2,6].

Activation of the phosphatidylinositol signaling pathway and cytosolic calcium oscillation-like phenomena appear to underlie phasic myometrial contractions; therefore one would anticipate that modulation of the activity of the IP_3R would have significant effects on myometrial contractions. The studies described in this report were performed to determine for the first time the effects of thimerosal on agonist-stimulated phasic myometrial contractions.

MATERIALS AND METHODS

For these studies, uteri were obtained from proestrus/estrus Sprague-Dawley white rats. After surgical removal, the uteri were rinsed in normal saline, placed in Earle's buffered saline solution (EBSS) (117 mM NaCl, 1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 5.6 mM glucose), and continuously aerated with 95% O_2 / 5% CO_2. *In vitro* isometric contraction studies were performed as previously reported from our laboratory [7,8]. Dose response studies were performed using oxytocin (0.48 mU/mL), aluminum fluoride (1.5 mM),

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potassium chloride (20 mM), ionomycin (1 μM), and Bay K 8644 (1 μM) with and without thimerosal (100-500 μM). To determine the role of the intracellular and extracellular calcium during thimerosal inhibition of contractile activity, additional studies were performed using oxytocin with thimerosal in nominal calcium-free buffer (ie. calcium-free EBSS), followed by the addition of calcium to physiologic concentrations. To determine the reversibility of the thimerosal effect on phasic contractions, washout experiments were performed by rapidly rinsing the muscle strips with warmed normal saline twice, then refilling the muscle baths with warmed, pre-aerated EBSS containing 5 mM DTT; after a subsequent washout and refill with warmed, pre-aerated EBSS, the strips were re-stimulated with oxytocin (0.48 mU/mL).

Total contractile activity was quantified by determination of the area under 5 minute contraction intervals; subsequently, the contraction data were normalized for tissue cross-section area, and reported as the percent of spontaneous or agonist-stimulated contractile activity as previously reported [7,8]. Statistical analysis was performed using Kruskal-Wallis one way analysis of variance on ranks and the Student-Newman-Keuls multiple comparisons test where appropriate (significance = $p < 0.05$).

RESULTS

As observed in Figure 1A, thimerosal over the concentration of 100-500 μM markedly inhibited phasic myometrial contractions stimulated in response to oxytocin (0.48 mU/mL). Quantitative studies confirmed that 250-500 μM thimerosal produced significant inhibition of these oxytocin-stimulated contractions compared to vehicle (distilled, deionized water) alone (Figure 1B). The washout experiments demonstrated that washout alone did not result in resolution of the thimerosal inhibition of contractile activity, or in the ability of oxytocin to re-stimulate myometrial contractions (data not shown). In contrast, washout followed by incubation of the muscle strips in buffer containing DTT (a thiol reducing agent) successfully reversed the thimerosal effect, resulting in the recurrence of spontaneous contractions, and the ability to stimulate the tissue again with oxytocin (Figure 2). Experiments performed utilizing nominal calcium-free buffer confirmed oxytocin-stimulated phasic contractions in response to calcium release from intracellular calcium stores alone (Figure 3). Thimerosal (150 μM) significantly inhibited these contractions, along with continued inhibition of contractile activity after the replacement of normal extracellular calcium concentrations, as also observed in Figure 3.

Aluminum fluoride stimulates a significant increase in phasic myometrial contractions through direct activation of the G-proteins coupled to PI-PLC [8]; comparable to its effects on oxytocin-stimulated contractions, thimerosal also significantly inhibited contractions stimulated in response to aluminum fluoride (Table 1). Previous reports have demonstrated that increased cytosolic calcium directly activates PI-PLC [9-11]. Increased phasic contractions in response to 20 mM KCl, ionomycin, and Bay K 8644 are thought to be produced by this effect, resulting in activation of cytosolic calcium oscillation-like phenomena. Phasic contractions produced by all of these agents were markedly inhibited by thimerosal, as demonstrated in Table 1.

DISCUSSION

Cytosolic calcium oscillations occur in multiple nonexcitable cell types, including smooth muscle myocytes, in response to stimulation by various hormones and neurotransmitters [12]. The phosphatidylinositol signaling pathway plays an important role during the generation of these intracellular calcium oscillations [9,12-14]. Receptor activated stimulation of phosphoinositide-specific phospholipase C (PI-PLC) results in increased production of IP_3; which leads to the release of

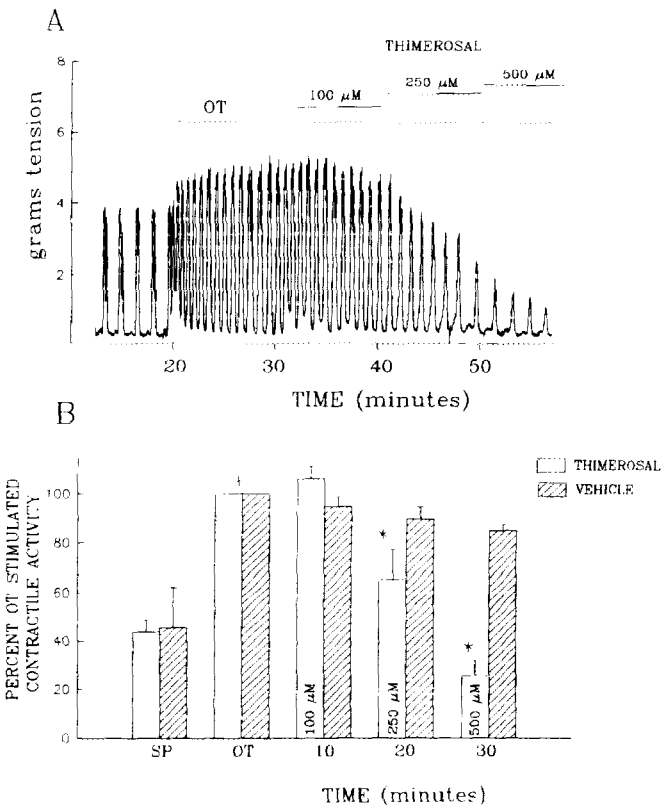

Figure 1.

Cumulative dose response effects of thimerosal on myometrial contractile activity. **A)** Contractile activity in grams tension generated during the spontaneous contraction period preceding 20 minutes, in response to 0.48 mU/mL oxytocin (OT), and the cumulative additions of thimerosal. **B)** Quantitative effects of thimerosal on oxytocin (OT)-stimulated contractions (as the percent of OT-stimulated activity). Time in minutes after OT stimulation. Each bar = mean ±S.D., N = 5 experiments. (+) $p<0.05$ for OT effect compared to spontaneous (SP) contractile activity; (*) $p<0.05$ for thimerosal treated strips compared to OT stimulation and vehicle controls.

sequestered intracellular calcium. Subsequently, cycles of release and refill of these intracellular calcium stores result in sustained oscillations of cytosolic calcium. Previously reported additional cellular events involved in these phenomena include the influx of extracellular calcium through membrane calcium channels, activation of calcium-induced calcium release (CICR), negative feedback by protein kinase C, and possible oscillations in IP_3 production [9,12-15].

Phasic smooth muscle contractions appear to occur simultaneously with repetitive transients (or oscillations) of cytosolic calcium [16-19]. Myometrial smooth muscle calcium oscillations appear to be similar, if not identical, to classic cytosolic calcium oscillations observed in other nonexcitable cells [20,21]. Previous reports from our laboratory have confirmed that activation of the phosphatidylinositol signaling pathway using various agonists including oxytocin, aluminum fluoride (a G-protein activator), potassium chloride (at concentrations of 10-30 mM), ionomycin (a calcium ionophore), and Bay K 8644 (a calcium channel agonist), result in the generation of phasic myometrial contractions [7,8,22-24]. Consistent with classic cytosolic calcium oscillation phenomena, agonist-stimulated phasic myometrial contractions were found to be markedly suppressed by enzyme

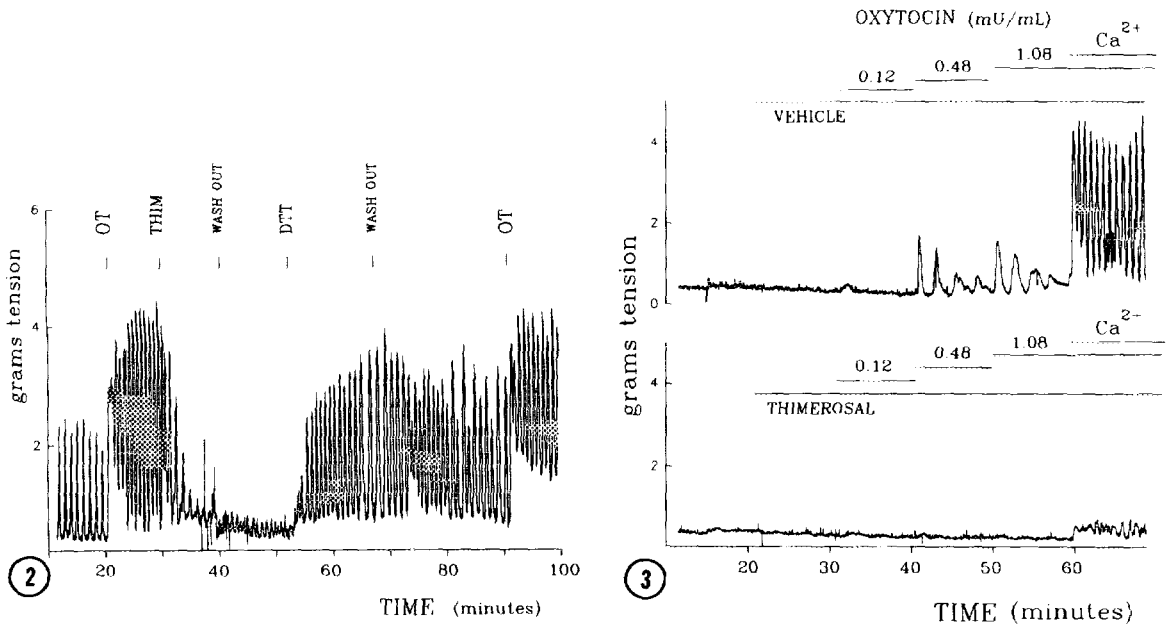

Figure 2.

Representative contraction study demonstrating DTT reversal of the thimerosal effect on oxytocin (OT)-stimulated contractions. Activity in grams tension generated during the spontaneous contraction period preceding 20 minutes, in response to OT (0.48 mU/mL), during the addition of thimerosal (500 μM), the buffer changes (wash out), the addition of DTT (5 mM), and in response to re-stimulation with OT.

Figure 3.

Representative experiments demonstrating the cumulative dose response effects of oxytocin (0.12 - 1.08 mU/mL) in the presence of 150 μM thimerosal (bottom) or a comparable volume of vehicle (top) in the nominal absence of extracellular calcium. Contractile activity in grams tension, time in minutes, and thimerosal or vehicle added at the 20 minute time point. At the 60 minute point, calcium chloride (Ca^{2+}) was added to produce a final calcium concentration of 1.6 mM.

inhibitors of PI-PLC, phorbol ester activation of protein kinase C, membrane permeant inhibitors of CICR, nifedipine inhibition of extracellular calcium influx, and complete removal of extracellular calcium using calcium-free buffer containing 2 mM EGTA [7,8,22-24].

Thimerosal, a sulphydryl reagent, has previously been reported to modulate the activity of the IP_3R in a dose related fashion: low thimerosal concentrations stimulate cytosolic calcium oscillations, whereas higher concentrations inhibit calcium oscillations, stimulate increased cytosolic calcium, and/or inhibit IP_3-induced calcium release [1-4,6]. Gericke et al. [4] demonstrated that 100 μM thimerosal inhibits over 90% of histamine-stimulated calcium release in endothelial cells; similarly, Sayer et al. [6] reported that 50 μM thimerosal almost completely inhibited IP_3-induced calcium release from cerebellar microsomes. These previously reported studies are consistent with the results observed in the present study; ie. thimerosal markedly inhibited agonist-stimulated phasic myometrial contractions, phenomena dependent upon both intracellular calcium release and extracellular calcium influx. The relatively higher concentrations of thimerosal required for these myometrial effects presumably represent less efficient penetration into intact tissue compared to isolated and/or permeabilized cells. Similar to the previous reports demonstrating

TABLE 1

THIMEROSAL EFFECTS ON AGONIST-STIMULATED PHASIC MYOMETRIAL CONTRACTILE ACTIVITY[a]

	Agonist	Thimerosal 100 μM	Thimerosal 250 μM	Thimerosal 500 μM	(N)
AlF_4^- (1.5 mM)	100±0.0	102.0±6.4	75.9±3.9	51.7±14.6*	(5)
KCl (20 mM)	100±0.0	101.3±21.8	48.2±25.6*	46.2±25.5*	(6)
Ionomycin (1 μM)	100±0.0	67.9±14.7*	28.4±14.6*	16.9±9.1*	(5)
BAY K 8644 (1 μM)	100±0.0	125.4±20.4	98.1±16.4	45.4±10.5*	(6)

a. phasic myometrial contractile activity in percent agonist stimulated activity; mean ± S.D.

(*) $p<0.05$ compared to agonist stimulated activity and to vehicle control studies.

DTT reversal of the effects of thimerosal [2,6], thimerosal-inhibition of agonist stimulated contractile activity was also found to be reversed using comparable concentrations of DTT. In summary, these observations have provided support for the hypothesis that calcium uptake and IP_3-induced calcium release are important events underlying agonist-stimulated phasic myometrial contractions.

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