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Thimerosal modulates the agonist-specific cytosolic Ca^{2+} oscillatory patterns in single pancreatic acinar cells of mouse

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Abstract Modulation of the agonist-specific cytosolic Ca^{2+} oscillatory pattern by thimerosal has been investigated in single pancreatic acinar cells using patch-clamp perforated whole-cell recording to measure the calcium-dependent chloride current ($I_{Cl(Ca^{2+})}$). 1 μM thimerosal, which fails to evoke Ca^{2+} oscillation alone, clearly changed the pattern of Ca^{2+} oscillation from pulsatile spikes (evoked by low concentrations of activators) to sinusoidal or transient oscillations. The mimetic action of thimerosal was independent of extracellular Ca^{2+}, was blocked by extracellular application of dithiothreitol or 10 mM caffeine, as well as by internal perfusion with heparin; but was unaffected by ruthenium red. We conclude that thimerosal modulates the agonist-specific cytosolic Ca^{2+} oscillatory patterns mediated by sensitizing the $InsP_3$-induced Ca^{2+} release.

Key words: Ca^{2+} oscillation; Inositol trisphosphate; Thimerosal; Pancreatic acinar cell; Ca^{2+}-dependent Cl^- current; Patch-clamp

1 Introduction

Intracellular calcium ions (Ca^{2+}) act as key second messengers that regulate many important functions of cells in a variety of living systems. In general, the Ca^{2+} signal is defined as an increase in cytoplasmic Ca^{2+} concentration which is generated either by release of Ca^{2+} from intracellular stores or by influx of extracellular Ca^{2+} through opening of Ca^{2+} channels in the plasma membrane. A wide variety of cells respond to biological activators with an oscillatory increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rather than a sustained increase [1,2]. These Ca^{2+} oscillatory patterns vary from one cell to another and even within individual cells responding to different agonists [1,3,4]. In pancreatic acinar cells, two major pancreatic acinar secretagogues, acetylcholine (ACh) and cholecystokinin (CCK), evoke clearly different oscillatory patterns of cytosolic Ca^{2+} signal in submaximal concentrations. Whether these two patterns are driven by similar mechanisms or not is still controversial [1,5–9]. Accumulating data indicate that the agonist-specific cytosolic Ca^{2+} oscillation patterns depend on receptor type, agonist concentration, intracellular inositol 1,4,5-trisphosphate ($InsP_3$) concentration, intracellular buffering, and resting $[Ca^{2+}]_i$ [7–9]. Resting $[Ca^{2+}]_i$ appears to play an important role in the determination of the agonist-specific cytosolic Ca^{2+} oscillatory patterns because in the high resting $[Ca^{2+}]_i$ condition, submaximal concentrations of ACh induced a typical CCK-like transient Ca^{2+} oscillation pattern rather than a sinusoidal one [9]. One critical hypothesis is cytosolic Ca^{2+} sensitized the $InsP_3$-induced Ca^{2+} release (IICR) [10].

The sulfhydryl (SH) reagent thimerosal has been reported to be able to sensitize IICR [11,12]. In single pancreatic acinar cells, high concentrations of thimerosal (5–100 μM) evokes cytosolic Ca^{2+} oscillations, and at low concentrations (1 μM), thimerosal potentiates the ACh elicited oscillation [13]. Here we provide new information that 1 μM thimerosal, which failed to evoke Ca^{2+} oscillation, mimics the Ca^{2+} oscillatory patterns (agonist-specific cytosolic Ca^{2+} oscillatory patterns) evoked by submaximal concentrations of ACh and CCK-8 at receptors, G-proteins or $InsP_3$ levels by measuring $I_{Cl(Ca^{2+})}$. The results indicate that the agonist-specific cytosolic Ca^{2+} oscillatory patterns depend on the sensitization of IICR.

2. Materials and methods

Experiments were performed on mouse single pancreatic acinar cells using patch-clamp whole-cell recording model to measure $I_{Cl(Ca^{2+})}$ [14,15]. Briefly, fragments of mouse pancreas were digested by collagenase (Wako Chem., 200 U/ml, 25–30 min, 37°C) in the presence of 1 mM Ca^{2+} to get single cells. The perforated patch-clamp whole-cell recording technique was used for the measurement of the transmembrane current [16]. With this method, the intracellular environment, especially cytosolic free Ca^{2+}, is not diluted by the pipette solution and intact intracellular function can be well maintained [17]. The standard extracellular solution contained (mM): NaCl 140, KCl 4.7, $CaCl_2$ 1.0, $MgCl_2$ 1.13, HEPES 10 and glucose 10 (pH 7.2). The perforated patch-pipette solution was (mM): KCl 150 and HEPES 10 (pH 7.2). Nystatin (Sigma) was dissolved in acidified methanol and the stock solution was diluted with the pipette solution to final concentration of 200 μg/ml just before recording. In internal perfusion experiments, a conventional whole-cell internal solution was employed (in mM): KCl 140, EGTA 0.25, $MgCl_2$ 1.13, Na_2ATP 5, glucose 10 and HEPES 10 (pH 7.2). All drugs used in the present experiments were from Sigma, except ruthenium red, which was from Wako Chemical. All experiments were carried out at room temperature (22–25°C).

3. Results

Since the typical agonist-specific cytosolic Ca^{2+} oscillation patterns occurred with an increase of agonist concentrations from suprathreshold to submaximum [7], it is pertinent to explain the reason that a specific oscillation pattern appears due to more intracellular $InsP_3$ formation [8]. Fig. 1a,b shows that similar patterns of pulsatile spiking are changed to typical oscillatory patterns (sinusoidal for ACh and transient for CCK-8) with increase of agonist concentrations from suprathreshold (ACh 10 nM, CCK-8 4 pM) to submaximum (ACh 50 nM, CCK-8 16 pM). However, increasing the concentra-

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tion of $InsP_3$ in pipette solution from 2 to 20 μM simply increased the frequency of short-lasting spikes. With further increase of $InsP_3$ to 100 μM, only the sustained response was observed (Fig. 1c–e). These results indicate that the agonist-specific cytosolic Ca^{2+} oscillatory patterns do not depend on cytosolic $InsP_3$ concentration.

In order to reveal the mechanism of the agonist-specific cytosolic Ca^{2+} oscillatory patterns, a specific agent which can mimic the Ca^{2+} oscillatory patterns is required. Fig. 2a shows a continuous recording from one cell in which 10 nM ACh always evoked repetitive spikes at a frequency that varied from cell to cell of 4 and 7 per min. Extracellular application of 1 μM thimerosal caused a sustained signal with a typical sinusoidal oscillation pattern after a 1–2 min latencies. The individual spikes in presence of thimerosal were considerably longer than those seen in the absence of thimerosal. In the same concentration of thimerosal, the similar short spiking response evoked by 4 pM CCK-8 was changed to transient oscillation (Fig. 2b).

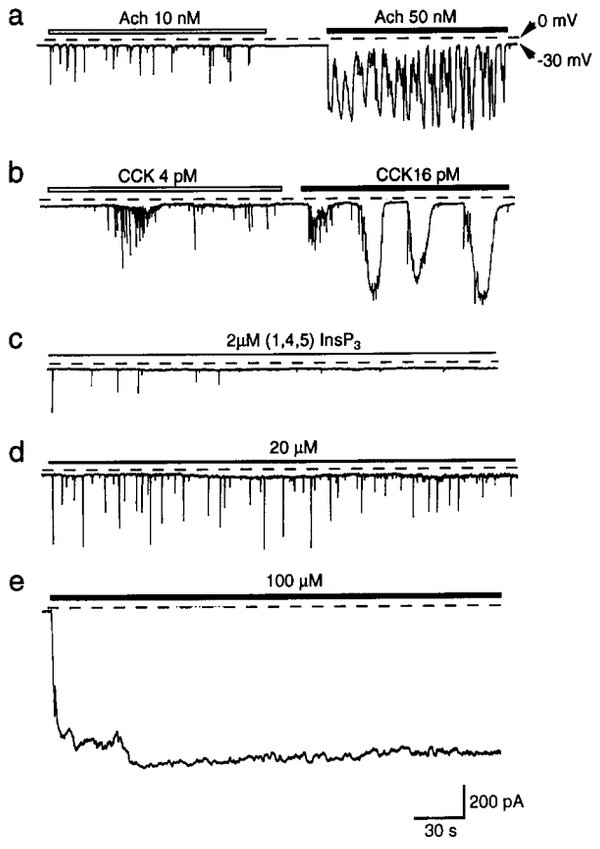

Fig. 1. Agonist-specific cytosolic Ca^{2+} oscillation patterns. Traces of whole-cell current (a–d) from different single mouse acinar cells. Acinar cells were voltage-clamped at a holding potential of −30 mV. The dashed horizontal line represents the current present at the 0 mV level, at this level no obvious current response was seen as this was close to the Cl^- equilibrium potential ($[Cl^-]_i = 150$ mM and $[Cl^-]_o = 147$ mM). Submaximal concentrations of ACh and CCK elicit typical sinusoidal and transient oscillations, respectively (a and b). Internal perfusion with low concentrations of $InsP_3$ evoke repetitive short-lasting oscillations (c and d). At high concentration, the $InsP_3$ induced response consisted of an initial spike followed by a sustained component (e). Agonists were applied for the periods indicated by the bar above each response. Each trace is a typical case from 3–5 cells.

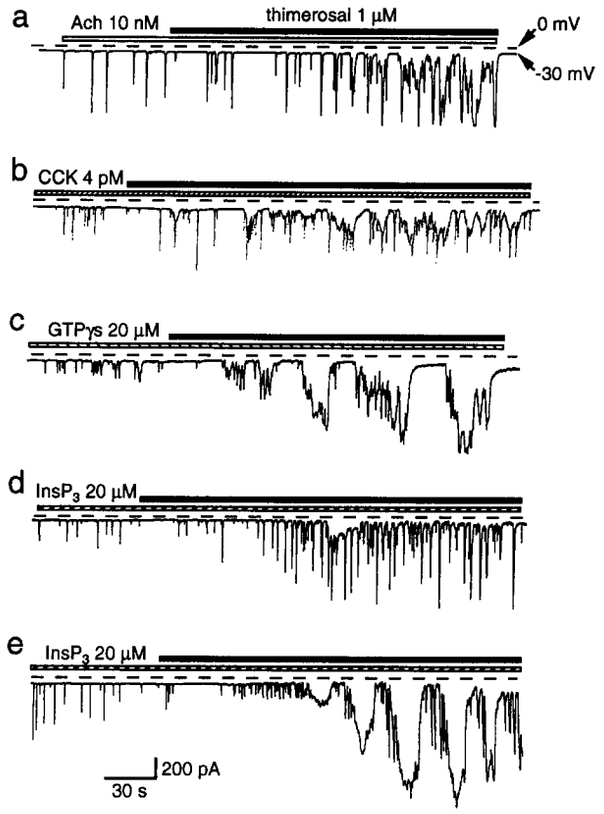

Fig. 2. Thimerosal mimicked agonist-specific cytosolic Ca^{2+} oscillatory patterns at the levels of membrane receptors, G-proteins, and $InsP_3$. Thimerosal potentiated the oscillatory responses evoked by low concentrations of ACh, CCK, GTPγs and InsP3, and changed the oscillatory patterns from pulsatile spiking to sinusoidal (a, d) or transient (b, c and e) oscillation. Agonists and thimerosal (black bars) were applied for the periods indicated by the bars above each response. a–e are from different cells and each trace is a typical case from 4–6 cells.

ACh and CCK bind to their membrane receptors, activate G-proteins and then produce $InsP_3$. $InsP_3$ then binds to intracellular receptors inducing Ca^{2+} release from the $InsP_3$-sensitive Ca^{2+} pool. Fig. 2c shows that the mimetic effect of thimerosal also occurs with direct perfusion of the G-protein agonist, GTPγs. One to two minutes following addition of thimerosal, the short-lasting spikes elicited by 20 μM GTPγs were changed to a typical transient oscillatory pattern represented by longer and larger waves, thereafter spike-wave complexes separated by silent intervals. With regard to the major action of thimerosal sensitizing the $InsP_3$ receptor/channel [11,12,18], we examined whether thimerosal mimicked the agonist-specific cytosolic Ca^{2+} oscillatory patterns mediated by increasing $InsP_3$ receptor/channel sensitivity. If so, thimerosal should mimic the agonist-specific cytosolic Ca^{2+} oscillatory patterns at the $InsP_3$ level. As shown in Fig. 2d,e, the pulsatile spiking evoked by internal perfusion with 20 μM $InsP_3$ is changed to sinusoidal ($n = 4$) or transient oscillations ($n = 3$), demonstrating that thimerosal indeed mimics the agonist-specific cytosolic Ca^{2+} oscillatory patterns by increasing the sensitization of the $InsP_3$ receptor/channel.

The two major Ca^{2+} release channels in cytosolic Ca^{2+} stores are on the endoplasmic reticulum and the sarcoplasmic reticulum. One is sensitized by $InsP_3$, which mediates IICR,

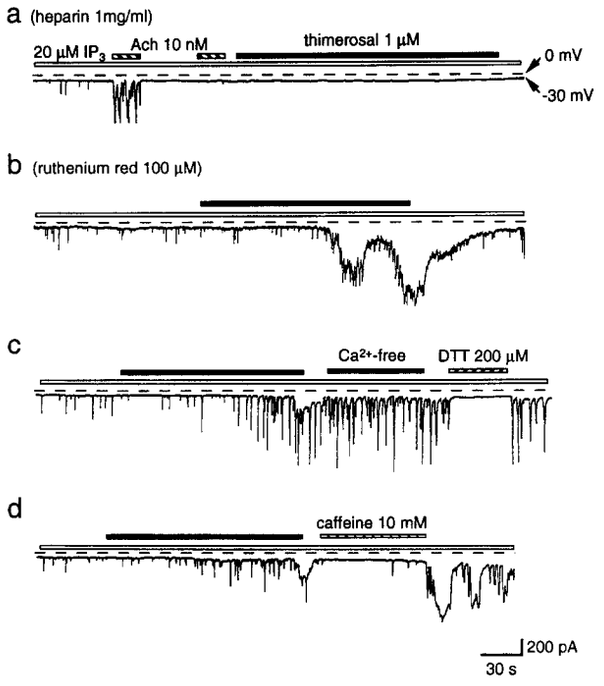

Fig. 3. Effects of heparin, ruthenium red, DTT, caffeine and extracellular Ca^{2+} on thimerosal modulated Ca^{2+} oscillation evoked by internal perfusion of $InsP_3$. (a) Internal perfusion with heparin blocked the Ca^{2+} oscillation evoked by $InsP_3$ and ACh, and abolished the potentiating action of thimerosal ($n=3$). (b) Internal perfusion with ruthenium red did not block the influence of thimerosal on $InsP_3$-evoked Ca^{2+} oscillation ($n=4$). (c) Thimerosal potentiated Ca^{2+} oscillation was blocked by 200 μM DTT ($n=3$), but not by Ca^{2+}-free extracellular solution ($n=3$). (d) The potentiated action of thimerosal was also blocked by 10 mM caffeine ($n=3$).

and another is not sensitized by $InsP_3$, and mediates Ca^{2+}-induced Ca^{2+} release (CICR). Pharmacological means are available to distinguish these two separate types of Ca^{2+} release channels [19]. IICR is inhibited by heparin, whereas this substance has no effect on the CICR [20,21]. CICR is inhibited by ruthenium red, which has no effect on the IICR [20,22]. To test whether thimerosal mimicked the agonist-specific cytosolic Ca^{2+} oscillation patterns mediated by IICR or CICR, we have employed intracellularly heparin and ruthenium red. Fig. 3a shows the result of one experiment, in which 1 mg/ml heparin was present in the pipette solution. Several minutes after establishment of the whole-cell recording, both the $InsP_3$ (20 μM) and the ACh (10 nM) evoked spiking responses were completely blocked. Under this condition, 1 μM thimerosal for at least 3 min was not able to potentiate any response in all three examined cells. After 3 min internal perfusion of ruthenium red (100 μM), which did not block the spiking response evoked by $InsP_3$, thimerosal still showed the mimetic effect (Fig. 3b). These data indicate that thimerosal mimics the agonist-specific cytosolic Ca^{2+} oscillatory patterns mediated by sensitizing IICR. In addition, we also found that the mimetic effect was independent of extracellular Ca^{2+}, and could be reversibly blocked by the SH-group-reducing agent, dithiothreitol (DTT) (Fig. 3c), as well as by a high concentration (10 mM) of caffeine (Fig. 3d).

4. Discussion

Our results show, for the first time, that thimerosal mimics the agonist-specific cytosolic Ca^{2+} oscillatory patterns in single pancreatic acinar cells using perforated patch whole-cell recording to measure $I_{Cl(Ca^{2+})}$. A major disagreement about agonist-specific cytosolic Ca^{2+} oscillation patterns is whether they are mediated by the same or separate mechanisms [1,5–7]. The results represented here indicate that the different Ca^{2+} oscillatory patterns normally induced by ACh and CCK may not be caused by separate mechanisms. First, at low concentrations, ACh and CCK-8 evoked quite similar short-lasting spikes, which were similar to the pulsatile spiking induced by internal perfusion with $InsP_3$ (Fig. 1a–d). This suggests that low concentrations of ACh, CCK and $InsP_3$ activate a common transduction pathway leading to Ca^{2+} release from Ca^{2+} stores, which are close to the plasma membrane. Second, low concentration (1 μM) of thimerosal mimics the agonist-specific cytosolic Ca^{2+} oscillatory patterns. This indicates that the activation of membrane receptors by submaximal concentrations of ACh and CCK may be mediated by changing the sensitization of the $InsP_3$ receptor/channel rather than simply increasing $InsP_3$ production [8] because in the present experiments, increases of $InsP_3$ concentration from 2 to 20 or 100 μM did not mimic the agonist-specific cytosolic Ca^{2+} oscillatory patterns (Fig. 1c–e). This idea is further confirmed by the finding that thimerosal changed the oscillation pattern during internal perfusion with $InsP_3$ from pulsatile spikes to sinusoidal in some cells (Fig. 2d) or transient oscillations in other cells (Fig. 2e). A possible explanation for these different responses is that these cells exhibit different resting Ca^{2+} level which might result in different IICR sensitivities [23–26]. Finally, the effects of thimerosal can be blocked by heparin and high concentration of caffeine, suggesting that the sensitization of the IICR may play a key role in determining the agonist-specific cytosolic Ca^{2+} oscillation patterns.

Thimerosal's effects do not appear to be mediated through any change in ACh or CCK receptors because the spiking response evoked by direct stimulation of G-proteins is also enhanced by thimerosal. Furthermore thimerosal does not appear to act on G-proteins because the same effects are seen in pulsatile spiking elicited by direct intracellular perfusion of $InsP_3$. In unfertilized hamster eggs, thimerosal sensitized CICR and caused Ca^{2+} oscillations [27]. Here, thimerosal is also unlikely to exert its effect through an increase in the sensitivity of the CICR mechanism since a CICR blocker, ruthenium red did not prevent thimerosal's influence, but heparin, a $InsP_3$ receptor/channel blocker, completely blocked the thimerosal's effect. The present data, therefore, show that thimerosal sensitizes the $InsP_3$ receptor/channel and modulates Ca^{2+} release from the $InsP_3$-sensitive Ca^{2+} pool.

The structure and function of most cysteine-containing proteins depend dramatically on the oxidation state of the SH groups. There is evidence that reagents which oxidize SH groups mobilize Ca^{2+} thereby increasing $[Ca^{2+}]_i$ on platelets, hepatocytes, oocytes and insulin-secreting cells [11,27–29]. In mouse pancreatic acinar cells, thimerosal mobilised Ca^{2+} from the $InsP_3$-sensitive intracellular pool and caused repetitive Ca^{2+} spikes [13]. In the present study, thimerosal appears to sensitize the $InsP_3$ receptor/channel and mimicked agonist-specific cytosolic Ca^{2+} oscillation patterns through an action on SH groups since its effects were prevented by the SH-group-reducing agent DTT. It has been reported that DTT not only inhibits thimerosal-induced Ca^{2+} oscillations, but

also ACh- and CCK-evoked responses [18], implying that agonist-specific cytosolic Ca^{2+} oscillation patterns might be related to the oxidation state of the sulfhydryl groups in the $InsP_3$ receptor/channel.

Although the effect of high concentrations of caffeine on thimerosal-evoked Ca^{2+} release from stores is obscure [13,29], our data clearly shows that 10 mM caffeine rapidly inhibits thimerosal's effect. Caffeine, as a classic CICR potentiator, enhances Ca^{2+} oscillations evoked by receptor activation or internal perfusion of $InsP_3$ at low concentrations (about 1 mM) [15]. At high concentrations (higher than 10 mM), however, caffeine can act as a functional antagonist of $InsP_3$ generation and inhibit agonist-induced generation of $InsP_3$ [30].

Stimulation of $InsP_3$ pathway evoked cytosolic Ca^{2+} increase may be mediated by Ca^{2+} release from intracellular pools or by influx of extracellular Ca^{2+}. The potentiation of $InsP_3$-evoked Ca^{2+} spiking by thimerosal is independent of extracellular Ca^{2+}, which indicates that thimerosal does not affect the Ca^{2+} transport system in plasma membrane.

According to previous studies [7–9] and our results, we conclude that IICR, especially the sensitized condition of the $InsP_3$ receptor/channel, contribute an important role in producing the agonist-specific cytosolic Ca^{2+} oscillatory patterns of pancreatic acinar cells.

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