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Thimerosal causes calcium oscillations and sensitizes calcium-induced calcium release in unfertilized hamster eggs

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Calcium-induced-calcium-release (CICR) was assayed in unfertilized golden hamster eggs by injecting Ca^{2+} and monitoring Ca^{2+}-dependent hyperpolarizing responses (HRs) and Ca^{2+}-sensitive fluo-3 fluorescence. Incubating eggs in the sulfhydryl reagent thimerosal caused $[Ca^{2+}]_i$ oscillations as monitored by Ca^{2+}-dependent HRs and decreased approximately 10-fold the Ca^{2+} injection current required to generate an HR and cause a large intracellular Ca^{2+} increase. Thimerosal also enhanced the sensitivity of eggs to Ca^{2+} injection in a calcium-free medium. The effects of thimerosal on CICR were prevented by dithiothreitol and were not mimicked by injecting inositol 1,4,5-trisphosphate. The data suggest that thimerosal may be an alternative agent for studying CICR in caffeine-insensitive cells.

Calcium ion release, Hamster egg, Calcium activated potassium conductance, Sulfhydryl reagent

1. INTRODUCTION

Calcium-induced release of intracellular Ca^{2+} (CICR) occurs in a wide variety of cells and plays a key role in generating oscillations of intracellular calcium $[Ca^{2+}]_i$ that occur in response to hormonal stimuli [1–4]. In muscle and nerve cells CICR is explained by the existence of a 400 kDa Ca^{2+}-activated Ca^{2+} release channel in the sarcoplasmic, or endoplasmic reticulum [5,6]. The frequency and open time probability of this channel can be enhanced by caffeine [7]. As a result of the enhanced CICR, caffeine causes intracellular Ca^{2+} release and $[Ca^{2+}]_i$ oscillations in muscle and nerve cells [8,9]. However, caffeine does not effect Ca^{2+} release in many cells that nevertheless do display both CICR and agonist stimulated $[Ca^{2+}]_i$ oscillations [1,10,11]. The lack of effect of caffeine has presented a problem in understanding how CICR operates in these caffeine-insensitive cells [1,2].

CICR has been demonstrated to occur in unfertilized hamster eggs [12]. The sensitivity of CICR in hamster eggs increases 10-fold after sperm-egg fusion and this leads to a series of transient oscillations in $[Ca^{2+}]_i$ [12–14]. These fertilization-induced $[Ca^{2+}]_i$ oscillations can be easily monitored in hamster eggs because each rise in $[Ca^{2+}]_i$ activates a plasma membrane potassium conductance which causes a hyperpolarizing membrane response (HR) [13,14]. In hamster eggs, as in other somatic cells, the phenomenon of CICR exists but it has not been easily studied or explained because caffeine does not trigger HRs, or affect CICR [15].

Recently it has been shown that the sulfhydryl reagent thimerosal causes $[Ca^{2+}]_i$ increases in platelets and leucocytes [16,17]. These observations suggested that sulfhydryl groups are involved in a Ca^{2+} release mechanism [16]. Here, I show that thimerosal causes a series of Ca^{2+}-dependent HRs in unfertilized hamster eggs. Furthermore, thimerosal mimics the action of sperm and enhances the sensitivity of the CICR mechanism. These data demonstrate that thimerosal is an alternative pharmacological probe for CICR in a caffeine-insensitive cell and suggest the existence of an analogous but distinct type of CICR to that characterized in muscle cells.

2. MATERIALS AND METHODS

All experiments were performed on zona free golden (Syrian) hamster eggs which were obtained and handled as described previously [12,18]. Eggs were bathed in M2 plus 4 mg/ml bovine serum albumin or else in a Ca^{2+}-free manganese-containing solution [12]. Thimerosal (sodium ethylmercurithiosalicylate), and other sulfhydryl reagents were dissolved directly into the appropriate media. Inositol 1,4,5-trisphosphate was dissolved at 1 mM in a KCl buffer [18]. Chemicals were from Sigma Chemicals (Poole, UK).

Membrane potentials were recorded with 3 M KCl-filled micropipettes. Ca^{2+} injections were made iontophoretically with

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Abbreviations HR, hyperpolarizing membrane potential response, CICR, calcium-induced-calcium-release, Ca^{2+}, calcium ions, $[Ca^{2+}]_i$, intracellular free calcium concentration; NEM, *n*-ethylmaleimide, $InsP_3$, inositol 1,4,5-trisphosphate, DTT, dithiothreitol

positive current pulses of 1 s duration applied through a second broken-tipped pipette that was inserted into the egg and filled with 0.5 M $CaCl_2$ plus 0.02% Nonidet P40 (NP40, Sigma, UK). Inclusion of NP40 in the Ca^{2+} injection pipette prevented the blockage of tips [12,19]. If insertion of the Ca^{2+}-injection pipette produced an HR, 2–3 min were allowed for the egg to recover before commencing the iontophoretic injections [12]. To measure membrane resistance, hyperpolarizing current pulses were applied through the voltage recording pipette in bridge balance mode or through the Ca^{2+}-injection pipette.

Relative $[Ca^{2+}]_i$ was monitored with the fluorescent calcium-sensitive dye fluo-3 [20]. Eggs were loaded with fluo-3 by incubating them for 1 h in M2 containing 4 mg/ml polyvinylalcohol and 40 μM fluo-3-AM plus 0.02% pluronic F-127 (Molecular Probes, USA). Cell fluorescence was measured using a Leitz Diavert epifluorescence microscope equipped with a Leitz I2 fluorescein filter block and a 9924B photomultiplier tube (Thorn EMI, UK). Fluorescence of fluo-3 loaded eggs was 5–10 times higher than that of cell autofluorescence.

3. RESULTS

Ca^{2+} release in hamster eggs was assayed by monitoring the Ca^{2+}-dependent HRs [14]. A variety of sulfhydryl reagents such as *N*-ethylmaleimide (NEM), *p*-hydroxymercuribenzoate and mersalyl, either had no effect or else caused a marked fall in membrane potential resistance that precluded further studies of their effects in hamster eggs (data not shown). However, thimerosal had effects at concentrations that did not effect the membrane in this way. Fig. 1A shows that perfusing medium containing 100 μM thimerosal into the bath containing an unfertilized hamster egg caused several repetitive HRs. The number of HRs varied considerably with different eggs and 4.1 ± 2.5 (mean and SD, n = 10) HRs occurred in the first 30 min after thimerosal addition. Lower concentrations of 20 μM thimerosal failed to cause any HR for up to 30 min (6 eggs). Higher concentrations (500 μM–1 mM) triggered an initially high frequency of HRs (data not shown), followed by a fall in membrane potential and membrane resistance.

HRs at fertilization in hamster eggs are associated with an increase in the sensitivity of the CICR mechanism which appears to cause the $[Ca^{2+}]_i$ oscillations [12]. To assay the sensitivity of CICR in control and thimerosal-treated eggs, Ca^{2+} was injected iontophoretically. Fig. 1B shows that 1 s current pulses of 0.5–5 nA Ca^{2+} into unfertilized control (untreated) hamster eggs caused HRs [12,21]. As has been found previously [12], a threshold injection current of 2.19 ± 0.5 nA (mean and SD) triggered an HR in 10/15 eggs that was regenerative [12]. Regenerative HRs were of similar magnitude to a maximal or full HR caused by a 5 nA × 1 s injection and were generally only obtainable once in each unfertilized egg [12,21].

Fig. 1C shows that after incubating eggs in 100 μM thimerosal for 10–30 min Ca^{2+} injection currents of only 0.1–0.2 nA (1 s) triggered full regenerative HRs. The injection current to trigger a full HR was 0.17 ± 0.09 nA in 14 different eggs (mean and SD). In

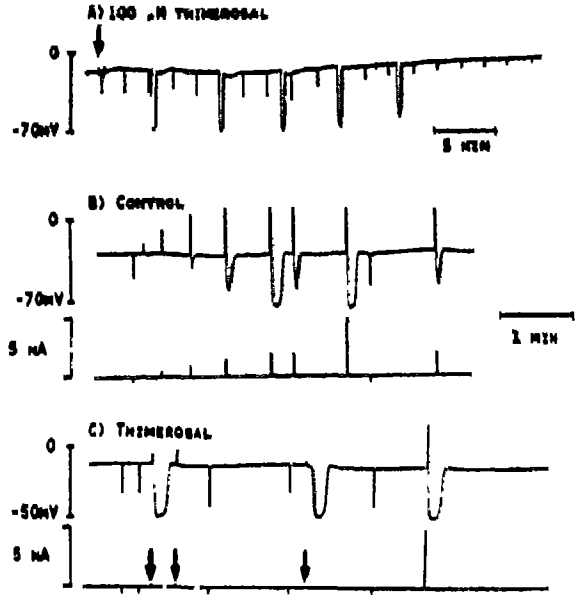

Fig. 1. Membrane potential recordings from unfertilized hamster eggs. (A) The egg was impaled with a voltage recording electrode and showed several HRs and a slight fall in membrane potential and resistance after perfusing 100 μM thimerosal into the bathing medium (at the arrow). Hyperpolarizing current pulses are 0.1 nA. (B) Control egg injected with positive current from the Ca^{2+}-containing pipette (indicated by upward deflections in the current record) of 0.2–5 nA × 1 s. A 2 nA pulse initially caused as large an HR as with 5 nA injection, a phenomenon only seen once in this egg. (Upward deflections of membrane potential caused by large Ca^{2+} injection are off-scale.) (C) An egg exposed to 100 μM thimerosal showing HRs after injecting Ca^{2+} current pulses of 0.2 nA and 0.1 nA (at the arrows) caused HRs that showed a refractory period and that were of equal magnitude to an HR caused by a 5 nA pulse. The time scale is the same in B–C.

thimerosal-treated eggs there was a short delay after injection in the Ca^{2+}-injection-induced HRs. The HRs could also be generated several times in the same egg, at intervals of 60–90 s, occasionally interspersed with unstimulated HRs. Although thimerosal at 20 μM did not induce spontaneous HRs, it did increase the sensitivity of eggs to Ca^{2+} injection and an injection current of 0.71 ± 0.5 nA (mean and SD, 10 eggs) triggered full HRs. Thimerosal is a sulfhydryl reagent and its effects appeared to involve sulfhydryl groups because incubating eggs in 100 μM thimerosal plus 5 mM DTT prevented the sensitization to Ca^{2+} and a mean injection current of 3.4 ± 1.2 nA (mean and SD, 10 eggs) was required to cause a full HR.

The above changes in sensitivity of eggs to Ca^{2+}-induced HRs are a true reflection of $[Ca^{2+}]_i$ increases because similar results were seen when relative $[Ca^{2+}]_i$ was monitored more directly with the fluorescent calcium indicator dye fluo-3. Fig. 2A shows that Ca^{2+} injection currents of 0.2–1.5 nA (1 s) into a control egg triggered small increases in fluo-3 fluorescence compared with a 5 nA Ca^{2+} injection. In contrast, in

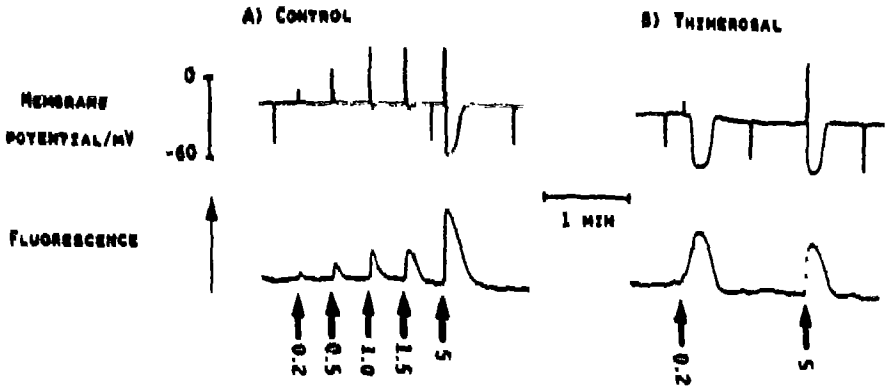

Fig 2 Simultaneous records of membrane potentials and fluo-3 fluorescence (in arbitrary units) from: (A) an egg injected with 0 2–5 nA Ca^{2+} injection current pulses and (B) an egg bathed in 100 μM thimerosal injected with a 0 2 and a 5 nA Ca^{2+} current pulse. Similar results were seen in 5 other control and 4 other thimerosal treated eggs Brief (1 s) hyperpolarizations were triggered by negative current pulses of 0.5 nA

the presence of 100 μM thimerosal the smaller Ca^{2+} injection of 0.2 nA caused a full HR and an increase in fluo-3 fluorescence similar to those from a 5 nA injection (Fig. 2B).

To determine if the action of thimerosal required external Ca^{2+} eggs were incubated in a calcium free medium containing 3 mM manganese (see [12]). Fig. 3A shows that small repetitive Ca^{2+} injections did not trigger HRs in control eggs bathed in a Ca^{2+}-free manganese medium. However, incubating eggs in 100 μM thimerosal in the calcium-free/manganese media sensitized eggs to Ca^{2+}-injection-induced HRs and repetitive 0.2 nA current pulses caused several HRs (Fig 3B).

To determine if thimerosal exerted any of its effects through increased $InsP_3$ production, the above experiments were compared to injecting $InsP_3$. In hamster eggs sustained $InsP_3$ injection through leakage from blunt pipettes causes a series of repetitive small HRs in which CICR may play a role [18]. Fig. 3C shows a small (0.2 nA) injection current of Ca^{2+} which induced an HR in an egg that was undergoing repetitive $InsP_3$-induced HRs. However, such HRs were partial HRs since they were always smaller than HRs triggered by maximal 5 nA calcium injection currents (Fig. 3C). $InsP_3$, therefore, did enhance CICR but its effects were distinct from those seen in thimerosal-treated eggs where Ca^{2+} injection triggered full HRs

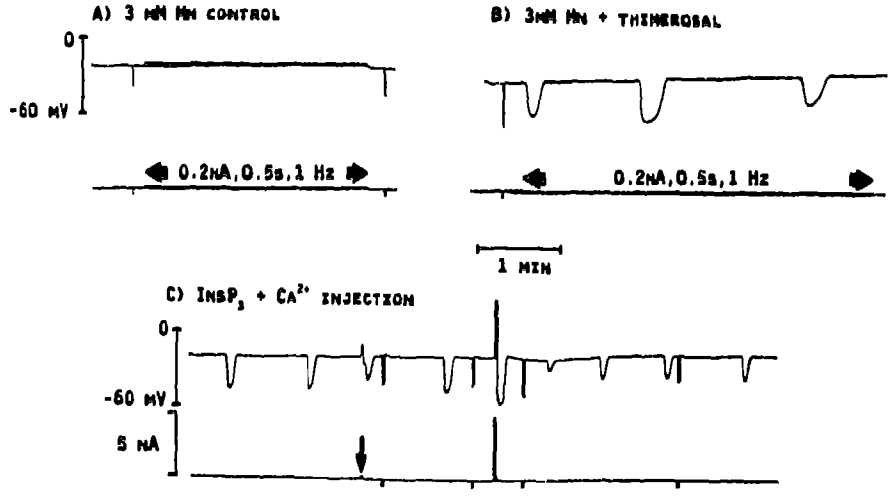

Fig 3 (A) Repetitive Ca^{2+} injection of 0 5 s × 0 2 nA pulses every second into a control egg in a calcium-free 3 mM manganese containing medium did not trigger HRs (B) Several HRs were triggered by the same Ca^{2+} injection pulses in an egg in calcium-free medium containing 100 μM thimerosal and 3 mM manganese (C) Shows an egg that was undergoing HRs after insertion of blunt $InsP_3$ containing pipette A Ca^{2+} injection current of 0 2 nA (at the arrow) caused a smaller HR than that seen with a 5 nA current pulse Records in A–C are each representative of at least 4 separate experiments

4. DISCUSSION

These data show that thimerosal increases the sensitivity of unfertilized hamster eggs to Ca^{2+}-injection-induced HRs by a factor of 5–20 times. As with fertilization of eggs, the increase in Ca^{2+} sensitivity leads to a series of Ca^{2+} oscillations as judged by the Ca^{2+}-dependent HRs. Thimerosal's effects do not appear to be mediated through any change in Ca^{2+} homeostasis. Thimerosal's effects did not rely on Ca^{2+} influx because a sensitization to Ca^{2+} occurred in a Ca^{2+}-free medium. Furthermore thimerosal did not appear to inhibit the various Ca^{2+} pumps in the egg because recovery times of $[Ca^{2+}]_i$ transients were similar in control and thimerosal-treated eggs. Agents that inhibit Ca^{2+} pumps cause an increase in the recovery time of individual HRs [19]. Thimerosal is also unlikely to exert its effects through a sustained rise in $[Ca^{2+}]_i$ or $InsP_3$ production since sustained Ca^{2+} injection causes a decrease in the sensitivity of the CICR mechanism [12], and sustained $InsP_3$ injection could not enhance Ca^{2+} sensitivity to the same extent as thimerosal. The present results, therefore, show that thimerosal specifically sensitizes CICR. This is the first indication that CICR can be pharmacologically enhanced in a caffeine-insensitive cell and suggests that thimerosal may be useful in probing CICR in other somatic cells that do not respond to caffeine.

Thimerosal appears to affect CICR through an action on sulfhydryl groups since its effects were prevented by DTT. It is not clear why thimerosal is more selective than other sulfhydryl reagents such as NEM, but the same selectivity of action is seen in platelets [16] However, thimerosal causes a more selective inhibition than NEM of the sodium pump by reacting with a particular subset of sulfhydryl groups [22]. The present results are consistent with the idea that thimerosal acts on sulfhydryl groups of protein involved in CICR. It would be useful to identify this protein It is not likely to be the 400 kDa ryanodine receptor since its presence in cells correlates with caffeine-induced Ca^{2+} release [23]. However, a novel 106 kDa calcium-activated-calcium release channel has been identified by its ability to react with, and be opened by, sulfhydryl reagents [24,25]. So far it has been isolated from sarcoplasmic reticulum of muscle, but there is evidence that a similar sulfhydryl gated Ca^{2+} channel exists in platelets and liver cells [26,27]. The same may also be true of hamster eggs

One effect of thimerosal in hamster eggs is that it mimics some of the changes at fertilization where there is a more sustained series of Ca^{2+} oscillations and an enhancement of CICR [12]. At fertilization the sperm may cause these changes by introducing a cytosolic protein factor directly into the egg cytoplasm after sperm-egg membrane fusion [29,30]. It is possible that the ultimate target of the sperm factor is the same putative Ca^{2+} release protein that is affected by thimerosal.

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REFERENCES

[1] Berridge, M J (1990) J Biol Chem. 265, 9583–9586.
[2] Jacob, R (1990) Biochim Biophys Acta 1052, 427–438
[3] Peterson, O H. and Wakui, M (1990) J Membr Biol 118, 93–105
[4] Goldbeter, A , Dupont, G. and Berridge, M J (1990) Proc Natl Acad Sci USA 87, 1461–1465
[5] Gill, D (1989) Nature 342, 16–18
[6] Lai, F A., Erikson, H P , Rousseau, E., Liu, Q.Y and Meissner, G (1988) Nature 331, 315–319
[7] Rousseau, E and Meissner (1989) Am J. Physiol 256, H328–333
[8] Kuba, K and Nishi, S (1976) J Neurophysiol 39, 547–562
[9] Herrmann Frank, A. (1989) J Muscl Res. Cell Motil 10, 437–445
[10] Jacob, R (1990) J Physiol 424, 29P
[11] Kong, S K , Choy, Y M., Fung, K P and Lee, C Y. (1990) J Physiol 424, 33P
[12] Igusa, Y and Miyazaki, S. (1983) J Physiol 340, 611–632
[13] Igusa, Y and Miyazaki, S (1986) J Physiol 377, 193–205
[14] Miyazaki, S. (1988) Dev Growth Diff 30, 603–610
[15] Miyazaki, S and Fujiwara, T. (1990) Japanese Physiol Soc Meeting, March 1990
[16] Hecker, M , Brune, B , Decker, K and Ullrich, V (1989) Biochem Biophys Res Commun 159, 961–968
[17] Hatzelmann, A , Haurand, M and Ullrich, V. (1990) Biochem Pharmacol 39, 559–567
[18] Swann, K , Igusa, I and Miyazaki, S (1989) EMBO J 8, 3711–3718
[19] Georgiou, P , Bountra, C , McNiven, A and House, C R (1987) Q J Physiol 72, 227–241
[20] Minta, A , Kao, J P Y and Tsien, R Y (1989) J Biol Chem 264, 8171–8178
[21] McNiven, A I , Yoshida, S , Georgiou, P and House, C R (1988) Pflugers Arch 412, 451–453.
[22] Kaplan, J H and Mone, M D (1985) Arch Biochem Biophys 237, 386–395
[23] Penner, R , Neher, E , Takeshima, H , Nishimura, S and Numa, S (1989) FEBS Lett 259, 217–221
[24] Zaidi, N F , Lagenaur, C F , Hilkert, R J , Xiong, H , Abramson, J J and Salama, G (1989) J Biol Chem 264, 21737–21747
[25] Zaida, N F , Lagenaur, C F , Abramson, J J , Pessah, I and Salama, G (1989) J Biol Chem. 264, 21725–21736
[26] Adunyah, S E and Dean, W L (1986) J Biol Chem. 261, 13071–13075
[27] Zhang, G H , Yamaguchi, M , Kimura, S., Higham, S and Kraus-Friedmann, N (1990) 265, 2184–2189
[28] Swann, K (1990) J Physiol 426, 63P
[29] Swann, K (1990) Development (in press)