Ca\(^{2+}\)-induced Ca\(^{2+}\) release in insulin-secreting cells

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The sulphydryl reagent thimerosal (50 µM) released Ca\(^{2+}\) from a non-mitochondrial intracellular Ca\(^{2+}\) pool in a dose-dependent manner in permeabilized insulin-secreting RINm5F cells. This release was reversed after addition of the reducing agent dithiothreitol. Ca\(^{2+}\) was released from an Ins(1,4,5)P\(_3\)-insensitive pool, since release was observed even after depletion of the Ins(1,4,5)P\(_3\)-sensitive pool by a supramaximal dose of Ins(2,4,5)P\(_3\) or thapsigargin. The Ins(1,4,5)P\(_3\)-sensitive pool remained essentially unaltered by thimerosal. Thimerosal-induced Ca\(^{2+}\) release was potentiated by caffeine. These findings suggest the existence of Ca\(^{2+}\)-induced Ca\(^{2+}\) release also in insulin-secreting cells.

Ca\(^{2+}\)-induced Ca\(^{2+}\) release; Sulphydryl reagent; Thimerosal; Intracellular Ca\(^{2+}\) transport; Insulin-secreting cell; Permeabilized cell

1. INTRODUCTION

In many cells, including insulin-secreting cells, receptor activation leads to phospholipase-C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in generation of diacylglycerol and inositol 1,4,5-trisphosphate, (Ins(1,4,5)P\(_3\))[1-3]. Ins(1,4,5)P\(_3\) is a well-known second messenger that mobilizes Ca\(^{2+}\) from specific intracellular stores, which appear to be structurally related to the endoplasmic reticulum. The Ins(1,4,5)P\(_3\)-sensitive pool is well established and comprises only a part of the intracellular non-mitochondrial Ca\(^{2+}\) pool [1]. The remainder of the pool, which is Ins(1,4,5)P\(_3\)-insensitive, is less well characterized. In a number of cell types it has been shown that Ca\(^{2+}\) can be released from part of the Ins(1,4,5)P\(_3\)-insensitive Ca\(^{2+}\)-pool by a rapid increase in intracellular free Ca\(^{2+}\)-concentration ([Ca\(^{2+}\)]\(_i\)). This Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) was initially identified in striated muscle cells and later has been demonstrated in a number of non-muscle cell types [5-9]. There are at present both theoretical grounds [10,11] and experimental evidence [12,13] to indicate that CICR may be important in the generation of Ca\(^{2+}\)-oscillations. However, CICR is difficult to demonstrate directly [4]. Ca\(^{2+}\) release evoked by two pharmacological agents, caffeine and ryanodine [5,14,15], is taken as evidence for the existence of CICR mechanism in cells. Caffeine is required to be used in millimolar concentrations and an optimal concentration can often not be used, because of its solubility limitations [16,17]. Ryanodine binds very slowly to its receptor [16,18].

Moreover, in many cells Ca\(^{2+}\) release cannot be demonstrated by caffeine or ryanodine [4,19-21]. It would, therefore, be useful to have other pharmacological tools that activate CICR in caffeine-insensitive cells [21].

The structure and function of most cysteine-containing proteins critically depend on the oxidation state of the protein's sulphydryl groups (SH-groups) [22,23]. There is evidence that the receptor-channel protein that mediates CICR (ryanodine receptor) in sarcoplasmic reticulum contains 'critical' SH-groups [24]. A number of sulphydryl reagents that oxidize SH-groups, release Ca\(^{2+}\) from sarcoplasmic reticulum by opening up the CICR channel [25-27]. Thimerosal is a sulphydryl reagent that has been demonstrated to be effective, in low micromolar concentrations, in releasing Ca\(^{2+}\) from intracellular pools in several non-muscle cell types [28-31]. More recently, it has been shown that thimerosal specifically sensitizes CICR in unfertilized hamster eggs and it has been suggested that this compound can be used to demonstrate CICR in caffeine-insensitive cells [21]. In this report, we demonstrate that thimerosal releases Ca\(^{2+}\) in permeabilized RINm5F cells, an effect potentiated by caffeine, suggesting the existence of CICR in insulin-secreting cells.

2. MATERIALS AND METHODS

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), all from Flow Laboratories (Scotland). Thimerosal (sodium ethylmercurithiosalicylate), ou-dithiothreitol (DTT), Ins(1,4,5)P\(_3\), caffeine, Ruthenium red and heparin were purchased from Sigma (St. Louis, USA). Ryanodine

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was obtained from Merck Sharp and Dohme (Rahway, NJ, USA). Calcium cocktail, containing neutral carrier ETH 1001 was from Fluka. All other chemicals were either from Sigma or Merck.

Cells were detached from culture flasks using trypsin-EDTA; washed twice with RPMI 1640 medium and twice with a cold nominally Ca²⁺-free buffer, containing 110 mM KCl, 10 mM NaCl, 2 mM KH₂PO₄, 1 mM MgCl₂, 0.5 mg/ml bovine serum albumin and 25 mM HEPES (pH 7.0 adjusted with KOH). Permeabilization was done by exposing cells to high-voltage electrical discharges (six pulses of 3.2 kV/cm). This treatment resulted in more than 90% permeabilized cells, as verified by Trypan blue uptake. After permeabilization, cell suspension was centrifuged and the pellet was kept on ice until use.

Eight µl of cell pellet was then added to a Plexiglass chamber containing 52 µl of incubation buffer. The incubation buffer was the same as the washing buffer, supplemented with 2 mM MgATP and an ATP-regenerating system, consisting of 10 mM phosphocreatine and 20 U of creatine kinase/ml. In addition, the incubation buffer also contained mitochondrial inhibitors consisting of 0.2 µM antimycin and 1 µg/ml oligomycin/ml. Additions were made from freshly prepared 100 times concentrated stock solutions. Thimerosal was dissolved in water or directly in the buffer. Changes in the ambient free Ca²⁺ concentration were recorded using a Ca²⁺-selective mini-electrode, constructed and calibrated essentially as described by Tsien and Rink [32]. None of the substances used in the study interfered with electrode function.

3. RESULTS

Permeabilized RINm5F cells (4.2×10⁷ cells/ml), in the presence of ATP and an ATP-regenerating system, sequestered Ca²⁺, resulting in a low steady-state buffer Ca²⁺ level. Addition of thimerosal (50 µM, final concentration) resulted in a rise in Ca²⁺ within 30 s, reaching a new increased steady state Ca²⁺ level in about 4 min (Fig. 1a). Thimerosal caused Ca²⁺ release in a dose-dependent manner (Fig. 1b). The smallest dose of thimerosal eliciting a detectable rise in Ca²⁺ was 25 µM and maximum release was obtained by 100 µM. Addition of the reducing agent DTT (2 mM, final concentration) resulted in immediate onset of re-uptake of Ca²⁺, eventually leading to complete resequestration of the ion (Fig. 1a).

Heparin binds to the Ins(1,4,5)P₃ receptor and inhibits Ins(1,4,5)P₃-induced Ca²⁺ release [33–36]. When added to the buffer in as high a dose as 1000 µg/ml, heparin did not inhibit Ca²⁺ release induced by 50 µM thimerosal (data not shown). When heparin was added at the end of completion of thimerosal-induced Ca²⁺ release, there was no re-uptake of the released Ca²⁺ (data not shown). Also, Ruthenium red (30 µM), a substance that has been shown to inhibit Ca²⁺-induced Ca²⁺ release [37], did not block thimerosal-induced Ca²⁺ release.

As shown in Fig. 2a, 20 µM (a maximal dose) of inositol(2,4,5)-trisphosphate (Ins(2,4,5)P₃), a non-metabolizable analogue of Ins(1,4,5)P₃ was used to empty the Ins(1,4,5)P₃-sensitive Ca²⁺ pool. Addition of thimerosal at the end of completion of Ins(2,4,5)P₃-induced Ca²⁺ release, resulted in further release of Ca²⁺. Similarly, when thimerosal was added at the end of completion of Ca²⁺ release by thapsigargin, there was additional release of Ca²⁺ (Fig. 2b).

When Ins(1,4,5)P₃ (5 µM, final concentration) was added after thimerosal-induced Ca²⁺ release was completed, marked additional increase in the release of Ca²⁺ was observed (Fig. 3). The magnitude of this Ins(1,4,5)P₃-induced Ca²⁺ release was essentially similar to that obtained by Ins(2,4,5)P₃ in the absence of thimerosal (cf. Fig. 2a).

Caffeine (2 mM, final concentration) did not induce
Ca\textsuperscript{2+} release (data not shown). Under our experimental conditions the maximal final concentration of caffeine that could be achieved by addition from stock solution was 2 mM. This was due to difficulty in obtaining a concentrated enough stock solution of caffeine [16,17]. Ryanodine (100 \mu M, final concentration) also did not induce Ca\textsuperscript{2+} release (data not shown). In the presence of caffeine (50 mM, final concentration), dissolved directly in the buffer, addition of thimerosal (50 \mu M) caused a more pronounced release of Ca\textsuperscript{2+} than in the absence of caffeine (cf. Fig. 4a and b).

4. DISCUSSION

The present study shows that the sulphydryl reagent thimerosal, in a dose-dependent manner, releases Ca\textsuperscript{2+} from an intracellular Ca\textsuperscript{2+} pool, in permeabilized RINm5F cells. The effect was completely reversed by addition of the reducing agent DTT, implying that the effect of thimerosal was specifically due to oxidation of SH-groups and not due to non-specific and permanent damage to the membrane of Ca\textsuperscript{2+}-storing vesicles. The effect of thimerosal in permeabilized RINm5F cells is not likely to reflect inhibition of intracellular Ca\textsuperscript{2+} pumps, since the sulphydryl reagent did not alter the initial rate of Ca\textsuperscript{2+} uptake. There is also evidence from other studies that thimerosal, in low concentrations, does not inhibit various Ca\textsuperscript{2+}-pumps [21,29]. Although several studies have demonstrated mobilization of Ca\textsuperscript{2+} from intracellular pools by thimerosal, its mechanism of action is not well understood [28–31]. It has been suggested that in non-muscle cells, sulphydryl reagents may induce Ca\textsuperscript{2+} release from the Ins(1,4,5)P\textsubscript{3}-sensitive pool, by sensitizing the Ins(1,4,5)P\textsubscript{3} receptor to endogenous levels of Ins(1,4,5)P\textsubscript{3} [38]. We and others have suggested that, under conditions of permeabilization, RINm5F cells also contain basal levels of Ins(1,4,5)P\textsubscript{3} [36,39]. In this study Ca\textsuperscript{2+} release is unlikely to be due to sensitization.
of the Ins(1,4,5)P₃ receptor to basal levels of Ins(1,4,5)P₃, as has been suggested [38,40], since prior presence of heparin in buffer, in as high a concentration as 1000 µg/ml, did not prevent the release. In fact, some studies have demonstrated that sulphydryl oxidation of the Ins(1,4,5)P₃ receptor rather inhibits Ins(1,4,5)P₃ binding [41] and Ins(1,4,5)P₃-induced Ca²⁺ release [42]. By contrast, other studies have shown that sulphydryl oxidation increases the sensitivity of the Ins(1,4,5)P₃ receptor to the trisphosphate [40]. Interpretation of such conflicting data is difficult because of the use of different sulphydryl reagents and various cell types involved. However, since sulphydryl oxidation may also sensitize CICR, it cannot be ruled out that the apparent increase in sensitivity to low doses of Ins(1,4,5)P₃ [40] may be confounded by CICR.

Clearly, thimerosal released Ca²⁺ from an Ins(1,4,5)P₃-insensitive pool and not from the Ins(1,4,5)P₃-sensitive pool, in RINm5F cells. This pool is not equivalent to mitochondria since mitochondrial blockers were routinely used. When the Ins(1,4,5)P₃-sensitive pool was depleted by a supramaximal dose of Ins(2,4,5)P₃, addition of thimerosal still released Ca²⁺, which in this case must have been released from an Ins(1,4,5)P₃-insensitive pool. In permeabilized RINm5F cells, we have demonstrated that thapsigargin [43] releases Ca²⁺ predominantly from an Ins(1,4,5)P₃-sensitive pool and empties the pool nearly completely (Islam and Berggren, unpublished data). Also, under these conditions, thimerosal released further Ca²⁺. The question whether thimerosal-induced Ca²⁺ release could be due to activation of CICR was not addressed in earlier studies [28-31], although one study attributed the release to an as yet unidentified intracellular Ca²⁺ transport system [29]. Convincing and direct evidence that thimerosal sensitizes CICR has come forth only recently [21]. In view of the present findings, it is likely that the thimerosal-induced Ca²⁺ release from an Ins(1,4,5)P₃-insensitive pool in RINm5F cells is due to opening up of CICR channel by SH-group oxidation. Evidence that thimerosal-induced Ca²⁺ release in RINm5F cells is due to activation of CICR is reinforced by our demonstration that it can be markedly enhanced by caffeine, which is known to activate or sensitize CICR.

The existence of CICR has been inferred from caffeine-induced Ca²⁺ release in a number of non-muscle cells [16,44-46]. It is possible that CICR occurs in many cell types but is not demonstrable, because of their apparent insensitivity to caffeine, as is the case with RINm5F cells. These cells may contain a variant of the 'classical' ryanodine receptor, which mediates CICR in sarcoplasmic reticulum. In view of recent demonstrations that CICR may be involved in the generation of Ca²⁺-oscillations and wave propagation [12,13], it is important to clarify its existence in different cells and in this context, thimerosal may be an additional tool. Although the physiological importance is far from being understood, this is the first report suggesting the existence of CICR in insulin-secreting cells.

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