Thapsigargin-induced Ca^{2+} mobilization in acutely isolated mouse lacrimal acinar cells is dependent on a basal level of Ins(1,4,5)P_3 and is inhibited by heparin

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The tumour-promoting agent thapsigargin has been shown to inhibit the microsomal Ca^{2+}-ATPase and cause Ca^{2+} mobilization in a variety of cell types including exocrine acinar cells [Bird, Obie and Putney (1992) J. Biol. Chem. **267**, 18382–18386]. When applied to acutely isolated lacrimal acinar cells, thapsigargin caused a slow biphasic activation of both the Ca^{2+}-dependent K^+ and Cl^- currents measured using the whole-cell patch-clamp technique. If the only action of thapsigargin is to inhibit sequestration into Ca^{2+} pools, then Ca^{2+} mobilization following exposure to thapsigargin indicates that there is a significant 'leak' of Ca^{2+} into the cytoplasm, which is normally countered by Ca^{2+}-ATPase activity. In the present study, we introduced the Ins(1,4,5)P_3 receptor antagonist heparin (200 μg/ml) into lacrimal acinar cells via the patch-clamp pipette. Following a 5 min preincubation in the presence of heparin, neither acetylcholine (1 μM) nor thapsigargin (1 μM) caused any significant increase in either Ca^{2+}-dependent current. Caffeine has been shown to suppress basal Ins(1,4,5)P_3 levels in exocrine acinar cells [Toescu, O'Neill, Petersen and Eisner (1992) J. Biol. Chem. **267**, 23467–23470]. Preincubation with caffeine (10 mM) also inhibited the response to subsequent exposure to thapsigargin. These data suggest that, in acutely isolated lacrimal cells, the source of the Ca^{2+} leak which gives rise to Ca^{2+} mobilization following inhibition of Ca^{2+} re-uptake by thapsigargin is Ca^{2+} release, from Ins(1,4,5)P_3-dependent Ca^{2+} pools, caused by resting Ins(1,4,5)P_3 levels.

INTRODUCTION

In exocrine acinar cells, stimulus–secretion coupling is achieved using raised intracellular Ca^{2+} as an intracellular second messenger. In response to agonist stimulation and membrane receptor-mediated activation of phospholipase C, the cleavage of phosphatidylinositol 4,5-bisphosphate generates Ins(1,4,5)P_3, which releases Ca^{2+} from intracellular stores. The sesquiterpene lactone thapsigargin has been shown to inhibit the microsomal ATPase in a variety of cell types and give rise to a slow increase in intracellular Ca^{2+} [1–6]. Studies in exocrine glands have indicated that the actions of Ins(1,4,5)P_3 and thapsigargin are not additive and therefore that thapsigargin mobilizes intracellular Ca^{2+} largely from Ins(1,4,5)P_3-dependent Ca^{2+} pools [5,6]. In recent years thapsigargin has gained acceptance as a tool with which Ins(1,4,5)P_3-dependent Ca^{2+} pools may be emptied in the absence of any increase in intracellular Ins(1,4,5)P_3 levels [7,8]. There is, however, one aspect of the mechanism by which thapsigargin increases intracellular Ca^{2+} that has previously been little considered. Preventing Ca^{2+} re-uptake into Ins(1,4,5)P_3-dependent Ca^{2+} pools is not by itself a sufficient mechanism to produce Ca^{2+} mobilization; there must also be Ca^{2+} 'leak' from these same pools. Under resting conditions, Ca^{2+} leak from intracellular pools must be precisely counterbalanced by re-uptake, but in the presence of thapsigargin, re-uptake is inhibited and leak from the intracellular pools slowly increases the intracellular Ca^{2+}. In the present study we investigate thapsigargin-induced Ca^{2+} mobilization in acutely isolated mouse lacrimal cells and present evidence that the leak from Ins(1,4,5)P_3-dependent Ca^{2+} pools is an Ins(1,4,5)P_3-mediated process.

MATERIALS AND METHODS

Adult male CD1 mice were killed by cervical dislocation and lacrimal cells were isolated by collagenase (Worthington Diagnostic U.S.A.) digestion [9]. Cells were allowed to attach to a plastic Petri dish and were viewed at $\times 400$ magnification. The whole-cell configuration was achieved with single cells using 1.5–2.0 MΩ patch-clamp pipettes pulled from Assistant haematocrit tubing using a DMZ pipette puller. Access resistance through the patch pipette was approximately three times that of the pipette itself. Cells were voltage clamped to -30 mV using the List EPC7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier. K^+ and Cl^- currents were measured separately by pulsing to 0 mV and -80 mV respectively for 100 ms twice per second [10]. Currents were digitized using the CED 1401 interface (Cambridge Electronics Design, Cambridge, U.K.) and stored and analysed using an IBM AT compatible computer with custom-written software [11]. The mean steady-state current elicited in response to each voltage step was calculated and these values were then plotted against time. All Figures except for 1(a) and 1(b) show data averaged from several experiments using a spreadsheet program. All of the data (collected at 2 Hz) from each experiment were averaged to derive these plots, and therefore there are 120 averaged values per min. Standard errors were calculated and displayed at appropriate intervals along the trace. These data were synchronized at the point of drug addition. Values in the text show means $\pm$ S.E.M. (n = number of experiments).

The patch-clamp pipette contained (in mM) 140 KCl, 1.13 $MgCl_2$, 10 glucose, 0.5 EGTA and 1 ATP, buffered to pH 7.2

Abbreviation used: ACh, acetylcholine.

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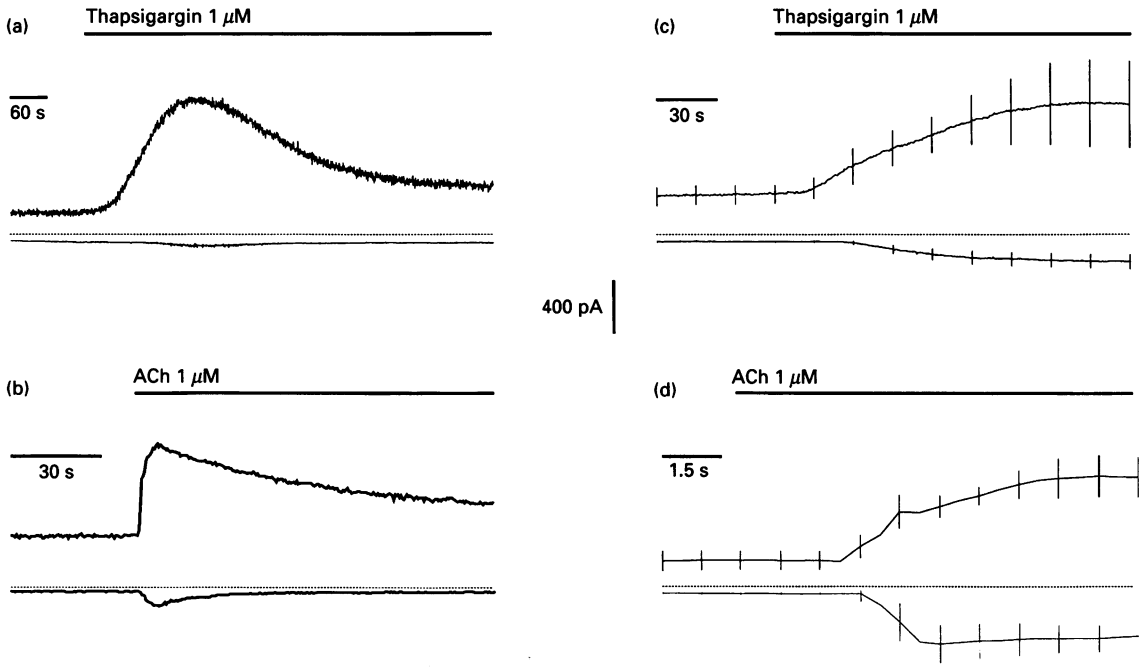

Figure 1 Biphasic activation of Ca^{2+}-dependent K^+ and Cl^- currents induced by agonist and thapsigargin

(**a**) Data from a single experiment showing K^+ (upper trace) and Cl^- (lower trace) currents in response to 1 μM thapsigargin measured over 15 min. The dotted line indicates the zero current level. (**b**) Data from a single experiment showing K^+ and Cl^- currents in response to 1 μM ACh measured over 2.5 min. (**c**) Averaged data from nine experiments showing the rising phase of the response of the K^+ and Cl^- currents to 1 μM thapsigargin measured over 4 min. (**d**) Averaged data from four experiments showing the rising phase of the response of the K^+ and Cl^- currents to 1 μM ACh measured over 10 s.

with 10 mM Hepes and, where indicated in the text and Figure legends, 200 μg/ml heparin. The external bathing solution contained (in mM) 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1.2 $CaCl_2$ and 10 glucose, buffered to pH 7.2 with 10 mM Hepes. Thapsigargin was dissolved in dimethyl sulphoxide (DMSO), which was present in the final solution at a concentration of 0.05%. DMSO alone was found to be without effect at concentrations of 1% (results not shown). Cells were superfused continuously at 0.5 ml/min from one of several parallel superfusion pipettes. The solution bathing the cell could be changed in 1–2 s. All experiments were carried out at 24±2 °C. These experiments take advantage of the access to the cell interior offered by the patch-clamp whole-cell technique to infuse membrane-impermeable substances into the cell.

RESULTS AND DISCUSSION

Figure 1(a) shows a typical response to thapsigargin recorded over 15 min. These data show the biphasic nature of the increased Ca^{2+}-dependent ion currents; the K^+ current declined from a peak of activation to a plateau within 5–10 min of exposure to thapsigargin. In several cases (as shown in Figure 1a), little or no sustained activation of the Cl^- current was observed. The biphasic pattern of Ca^{2+}-dependent currents in response to thapsigargin is similar to that seen in response to agonist. A typical response to 1 μM acetylcholine (ACh) is shown in Figure 1(b) for comparison. The time course of the biphasic increase in the K^+ current evoked by thapsigargin observed in the present study is also similar to the biphasic increase in intracellular Ca^{2+} reported in lacrimal cells by Kwan et al. [4]. The rising phase of thapsigargin-stimulated Ca^{2+}-dependent K^+ and Cl^- currents is shown in Figure 1(c). Following a 15–20 s lag, the K^+ current increased from 304±70 pA immediately before exposure to thapsigargin, to a peak of 995±318 pA measured 2.5 min later. The Cl^- current increased after 30–40 s from −53±7 pA to −194±53 pA over the same period. In lacrimal cells both the K^+ and the Cl^- channels are Ca^{2+}-dependent, but the K^+ channel is sensitive to Ca^{2+} in a lower concentration range than is the Cl^- channel [12–14]. Therefore Ca^{2+} mobilization following stimulation by ACh or brought about by inhibition of the microsomal ATPase by thapsigargin is seen first and most clearly as an increase in the whole-cell K^+ current [15] (Figures 1c and 1d). In this respect the patterns of response evoked by both agonist and thapsigargin are similar, the obvious difference being that the response to the latter is almost exactly 20 times slower Thapsigargin at 1 μM failed to activate the Ca^{2+}-dependent K^+ current in two out of 16 cell preparations, and these cells were discarded. The response to lower thapsigargin concentrations (0.1–0.5 μM) was variable; where there was a detectable activation of the K^+ current it usually occurred over an extended time period (> 10 min). There was no consistent enhancement of either the time course or the maximum K^+ current on increasing the thapsigargin concentration up to 2–5 μM.

Heparin is known to be a potent antagonist of the Ins(1,4,5)P_3 receptor [16,17] and to inhibit Ins(1,4,5)P_3-mediated activation of Ca^{2+}-dependent ion channels in a number of cell types, including exocrine acinar cells [18,19]. Figure 2(a) shows that, following infusion of 200 μg/ml heparin via the patch-clamp pipette, the response to ACh (1 μM) was almost completely inhibited. For comparison, in the absence of heparin, the peak response to 1 μM ACh (Figure 1d) was an increase in the K^+ current of 604±143 pA (n = 4; peak values measured 10 s after exposure to 1 μM ACh). These data demonstrate that there was good access to the cell interior via the patch-clamp pipette in these

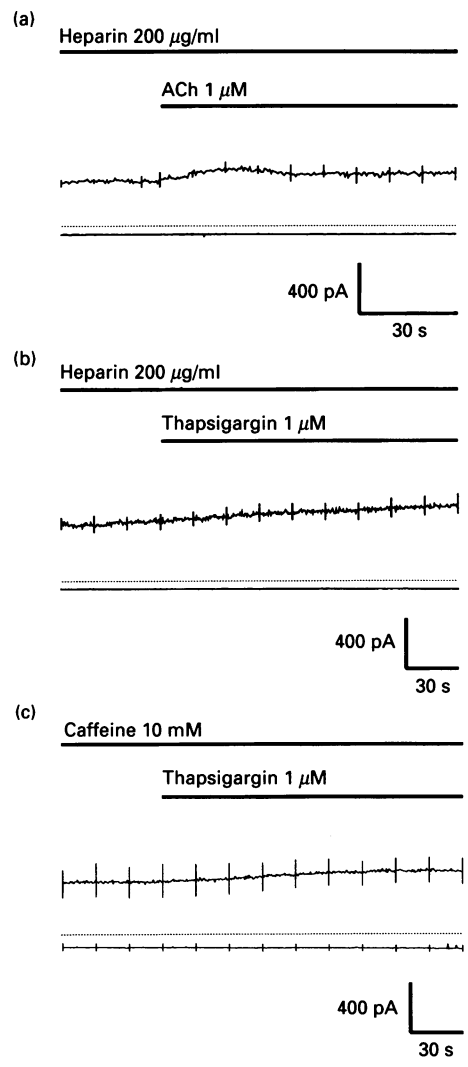

Figure 2 Inhibition of ACh- and thapsigargin-evoked Ca^{2+}-dependent ion currents by heparin and inhibition of thapsigargin-evoked Ca^{2+}-dependent currents by caffeine

(**a**) Averaged data from three experiments following a 3–5 min preincubation with heparin (200 mg/ml) in the intracellular medium (not shown) showing the K^+ current (upper trace) and Cl^- current (lower trace) recorded over a 90 s period following exposure to 1 μM ACh. The dotted line indicates zero current. (**b**) Averaged data from five experiments following a 5–8 min preincubation with heparin (200 μg/ml) in the intracellular medium (not shown) showing the K^+ current and Cl^- current recorded over a 3 min period following exposure to 1 μM thapsigargin. (**c**) Averaged data from five experiments following a 3–5 min preincubation with caffeine (10 mM) in the extracellular medium (not shown) showing the K^+ current and Cl^- current recorded over a 3 min period following exposure to 1 μM thapsigargin.

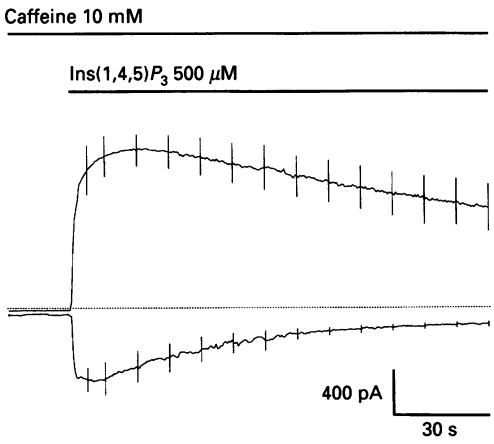

Figure 3 Lack of inhibition of Ins(1,4,5)P_3-evoked Ca^{2+}-dependent K^+ and Cl^- currents by preincubation with caffeine

Averaged data from five experiments following a 5–8 min preincubation with caffeine (10 mM) in the extracellular medium (not shown) while the cell was maintained in the cell-attached patch configuration. The upper trace shows the K^+ current and the lower trace the Cl^- current recorded over a 2 min period following breakthrough to the whole-cell configuration and release of 500 μM Ins(1,4,5)P_3 to the cell interior. The dotted line indicates zero current.

experiments and that heparin reached the Ins(1,4,5)P_3 receptor to prevent binding of Ins(1,4,5)P_3 generated by ACh.

Figure 2(b) shows that infusion of heparin (200 μg/ml) also inhibited Ca^{2+} mobilization caused by thapsigargin. This was an unexpected result, as it has previously been shown that thapsigargin has no effect on Ins(1,4,5)P_3 metabolism [7,8]. The inhibition of the thapsigargin response was specific to the active, Ins(1,4,5)P_3-receptor-binding form of heparin; the de-*N*-sulphated heparin analogue, which does not bind to Ins(1,4,5)P_3 receptors [16,20,21], was without effect (results not shown). The most straightforward explanation of this action of heparin is that, in acutely isolated lacrimal acinar cells, the leak of Ca^{2+} from Ins(1,4,5,)P_3-dependent Ca^{2+} pools, which is a necessary feature of the mechanism by which thapsigargin mobilizes intracellular Ca^{2+}, depends on a basal level of Ins(1,4,5)P_3. By blocking Ins(1,4,5)P_3 receptors with heparin, this component of the leak is no longer available and thus thapsigargin cannot mobilize intracellular Ca^{2+}.

In pancreatic acinar cells the methylxanthine caffeine has been shown to lower basal Ins(1,4,5)P_3 levels [22]. Figure 2(c) shows that preincubation with 10 mM caffeine inhibited thapsigargin-evoked Ca^{2+} mobilization. This is consistent both with a reduction of basal Ins(1,4,5)P_3 levels by caffeine in lacrimal acinar cells and with a role for basal Ins(1,4,5)P_3-dependent Ca^{2+} leak in the mechanism of action of thapsigargin. Caffeine has been previously shown to partially inhibit thapsigargin-induced Ca^{2+} mobilization in rat atrial cells [23].

The heparin- and caffeine-induced block of thapsigargin-evoked K^+ and Cl^- current activation did not stem from direct inhibition of the K^+ and Cl^- channels. The intracellular Ca^{2+} concentration can be elevated and K^+ and Cl^- currents activated without any possible role for Ins(1,4,5)P_3 by using the Ca^{2+} ionophore ionomycin. Neither 10 mM caffeine ($n = 3$) nor 200–400 μg/ml heparin ($n = 4$) had any inhibitory effect on ion channel activation following exposure to 1 μM ionomycin (results not shown).

An alternative mechanism by which caffeine could inhibit thapsigargin-evoked Ca^{2+} mobilization is via direct inhibition of the Ins(1,4,5)P_3 receptor, which would prevent the Ins(1,4,5)P_3-mediated Ca^{2+} leak without altering basal Ins(1,4,5)P_3 levels. The data in Figure 3 indicate that this is not the case. Previous studies have shown that a very high Ins(1,4,5)P_3 concentration is required to fully mobilize intracellular Ca^{2+} in acutely isolated lacrimal cells [9,14,24,25]. Preincubation with 10 mM caffeine did not prevent Ca^{2+} mobilization and activation of both the K^+ and the Cl^- currents by 500 μM Ins(1,4,5)P_3. For comparison, the control K^+ current in these experiments, which stabilizes in 1–5 s after breakthrough into the whole-cell condition, was 304 ± 70 pA,

and the peak K^+ current in the presence of Ins(1,4,5)P_3 was 1395 ± 137 pA. These values are statistically identical to our previously published data for the response to 500 μM Ins(1,4,5)P_3 without preincubation in the presence of caffeine [14]. Thus we did not observe any inhibition of the response to Ins(1,4,5)P_3 which would have been expected if caffeine was blocking Ca^{2+} efflux from the Ins(1,4,5)P_3-dependent Ca^{2+} pools by inhibiting the Ins(1,4,5)P_3 receptor.

One well documented effect of caffeine is to inhibit phosphodiesterase activity and increase cyclic nucleotide activity [26]. Cyclic AMP has been shown to inhibit Ins(1,4,5)P_3 metabolism [27–29] and to inhibit agonist-evoked repetitive transient K^+ and Cl^- currents in mouse submandibular cells [10]. However, preincubation with cyclic AMP or cyclic GMP in the concentration range 10 μM–1 mM or with membrane-permeable chlorophenylthio cyclic AMP (100 μM) in the extracellular medium had no detectable effect on Ca^{2+} mobilization induced by subsequent addition of thapsigargin (results not shown). Thus it seems unlikely that inhibition of phosphodiesterase activity and increased cyclic nucleotide levels are the means by which caffeine inhibits thapsigargin-mediated Ca^{2+} mobilization; how caffeine exerts this action remains unclear.

Acutely isolated acinar cells from exocrine glands have proved to be a useful model system for investigating intracellular signalling by raised intracellular [Ca^{2+}]. Studies using these cells have greatly contributed to our understanding of the processes by which intracellular Ca^{2+} is mobilized and how activation of Ca^{2+} influx is achieved. In the present study, thapsigargin-evoked Ca^{2+} mobilization in acutely isolated mouse lacrimal cells was completely blocked by heparin, indicating that the background Ca^{2+} leak from microsomal stores is an Ins(1,4,5)P_3-mediated process. It is likely that there are other Ca^{2+} leak pathways in these cells; however, in the presence of heparin or caffeine, these leak pathways alone are unable to elevate intracellular [Ca^{2+}] sufficiently to activate the Ca^{2+}-dependent K^+ current. The plasma membrane Ca^{2+}-ATPase will continue to function in the presence of thapsigargin, and thus Ca^{2+} extrusion could account for the inability to detect rises in intracellular [Ca^{2+}] due to any Ins(1,4,5)P_3-independent Ca^{2+} leak. Studies on other tissues, including one on mouse lacrimal cells maintained in primary culture and one on rat lacrimal cells, have shown little or no inhibition of thapsigargin-evoked Ca^{2+} mobilization by heparin [4,24,30–33]. Presumably the alternative sources of Ca^{2+} leak dominate in these cells. Species differences and effects of cell isolation techniques on basal rates of Ca^{2+} leak from intracellular Ca^{2+} pools have not been investigated, but may prove to be important if the determining factor in whether or not thapsigargin can mobilize Ca^{2+} in the presence of heparin is a balance between Ins(1,4,5)P_3-insensitive Ca^{2+} leak and Ca^{2+} extrusion.

Another important difference between mouse and rat lacrimal cells is their respective sensitivities to exogenous Ins(1,4,5)P_3. Acutely isolated mouse lacrimal cells require very high Ins(1,4,5)P_3 levels to be introduced before significant activation of Ca^{2+}-dependent currents is achieved [9,14,24,25]. The difference we now report in the nature of the Ca^{2+} leak may reflect another aspect of the same phenomenon.

This work was supported by a grant from the Wellcome Trust to D. V. G. We thank P. Thorn and O. H. Petersen for help and advice throughout this study.

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Received 2 August 1993/28 October 1993; accepted 4 November 1993