

Activation by calcium alone of chloride secretion in T₈₄ epithelial cells

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- 1 The goal of this study was to determine if an increase in cytoplasmic calcium concentration ($[Ca^{2+}]_i$), in the absence of additional second messengers derived from membrane phospholipid turnover, is a sufficient signal to induce chloride secretion across monolayers of the human colonic epithelial line, T₈₄.
- 2 Thapsigargin was used to increase $[Ca^{2+}]_i$ by inhibiting the endomembrane Ca^{2+} -ATPase. $[Ca^{2+}]_i$ was monitored in monolayers by fura-2 fluorescence spectroscopy, chloride secretion by measuring changes in short circuit current (I_{sc}) in modified Ussing chambers, and inositol phosphates were measured by radio-h.p.l.c. of extracts of cells prelabelled with [³H]-inositol.
- 3 Thapsigargin increased $[Ca^{2+}]_i$ and I_{sc} in parallel, without increasing any inositol phosphates. The effect of thapsigargin on I_{sc} was abolished by the intracellular calcium chelator, *bis*-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA).
- 4 Increasing $[Ca^{2+}]_i$ with thapsigargin did not prevent a subsequent calcium response to carbachol or histamine if extracellular calcium was available. In the absence of extracellular calcium, only one such release of calcium to hormonal stimulation occurred when cells were pretreated with thapsigargin, and a second response to either carbachol or histamine was essentially abolished.
- 5 Addition of carbachol or histamine to thapsigargin-treated cells mounted in Ussing chambers caused a transient further increase in I_{sc} followed by termination of the response, even though $[Ca^{2+}]_i$ continued to rise.
- 6 We conclude that an elevation in $[Ca^{2+}]_i$ is a sufficient signal to induce chloride secretion in T₈₄ cells. Rather than being required to stimulate secretory responses, additional second messengers induced by hormonal secretagogues (such as inositol phosphates) may in fact serve to limit the secretory response.

Keywords: Epithelium; chloride secretion; calcium; inositol phosphates; thapsigargin; carbachol; histamine

Introduction

Active secretion of chloride is thought to be an important driving force for secretion of fluid across a number of epithelial surfaces, including the lung and intestine (McCann & Welsh, 1990; Barrett & Dharmasathaphorn, 1991). Disregulation of this process occurs in a number of diseases, including secretory diarrhoea and cystic fibrosis. There are accordingly clinical as well as basic reasons for wishing to understand fully the control of chloride secretion. Studies with the transformed human colonic epithelial cell line T₈₄, which permits precise correlations of biochemical events with the physiological response of chloride secretion, have revealed three primary signal transduction pathways linking receptor occupancy to the final response of chloride secretion (Barrett & Dharmasathaphorn, 1990). These involve elevations in cellular levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP), guanosine 3':5'-cyclic monophosphate (cyclic GMP) or free cytosolic calcium ($[Ca^{2+}]_i$). However, particularly for the last of these named second messengers, the precise steps which constitute the coupling between stimulus and secretion have not been fully elucidated.

$[Ca^{2+}]_i$ is widely recognized to be involved in the control of chloride secretion in a number of cell types (Bolton & Field, 1977; Frizzell, 1977; Chase, 1984; Cartwright *et al.*, 1985; Dharmasathaphorn & Pandol, 1986; Donowitz & Welsh, 1986; Mandel *et al.*, 1986; Welsh, 1987; Wasserman *et al.*, 1988; Dharmasathaphorn *et al.*, 1989a; Wong *et al.*, 1989). However, a definition of its exact role in the process has been difficult because most hormones which elevate $[Ca^{2+}]_i$ also have numerous other biochemical effects on the cell. For

example, stimulation of T₈₄ cells with the prototype calcium-dependent chloride secretagogue, carbachol, results in phosphatidylinositol turnover, elevations in a number of phospholipid metabolites including inositol (1,4,5) trisphosphate, inositol (1,3,4) trisphosphate, inositol tetrakisphosphates, diacylglycerol and phosphatidic acid, synergistic enhancement of responsiveness to cyclic nucleotide-dependent stimuli, and phosphorylation of substrates of protein kinase C (Cartwright *et al.*, 1985; Cohn, 1990; Vajanaphanich *et al.*, 1993; Kachintorn *et al.*, 1993). Moreover, it has been postulated that some of the biochemical events, or others as yet unidentified, might be required for the effect of calcium on chloride secretion, and that calcium acting alone is insufficient to activate the secretory response (Dharmasathaphorn *et al.*, 1989a). The primary evidence for this hypothesis rests on the observation that there is a very poor correlation between the ability of various substances to increase $[Ca^{2+}]_i$ and stimulate chloride secretion. Calcium ionophores can have marked and sustained effects on $[Ca^{2+}]_i$ while being relatively ineffective as transient chloride secretagogues, whereas histamine and carbachol stimulate more marked chloride secretion in association with a modest rise in $[Ca^{2+}]_i$ (Dharmasathaphorn *et al.*, 1989a).

Part of the difficulty in fully delineating the role of calcium in chloride secretion across T₈₄ cells arose from the previous lack of an experimental means of elevating $[Ca^{2+}]_i$ in the absence of the biochemical changes described above. Calcium ionophores such as ionomycin and A23187, which have been widely employed as experimental tools in this regard, are now well-recognized to be capable of stimulating phospholipase C and consequent inositol phosphate metabolism. In the present study, we have employed another means of elevating cytoplasmic calcium concentrations, thapsigargin.

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This sesquiterpene lactone has been shown to induce a sustained $[Ca^{2+}]_i$ signal in many cells, including platelets, hepatocytes, parotid acinar cells and lymphocytes (Thastrup *et al.*, 1987; 1990; Takemura *et al.*, 1989; Mason *et al.*, 1991). The rise in calcium, which is derived from both intracellular and extracellular sources, is reportedly independent of inositol phosphate generation (Jackson *et al.*, 1988; Takemura *et al.*, 1989; Law *et al.*, 1990). It is thought to act directly on intracellular stores of calcium as an inhibitor of the endomembrane Ca^{2+} -ATPase (Thastrup *et al.*, 1990). Secondly, this agent also stimulates influx from the extracellular compartment, presumably because the internal stores are in some way linked to the plasma membrane calcium permeability pathway (Takemura *et al.*, 1989). We therefore reasoned that this might be a useful agent to settle the question of whether calcium, acting alone in the absence of products derived from membrane phospholipid turnover, was capable of stimulating transepithelial chloride secretion. We have also examined the possible links between the agonist (i.e. inositol (1,4,5) trisphosphate-) sensitive pool of calcium that is mobilized by agents such as carbachol or histamine in T_{84} cells, with the calcium pool that is mobilized by thapsigargin.

Methods

Measurement of Cl^- secretion and $[Ca^{2+}]_i$

Growth and maintenance of the human colonic epithelial cell line, T_{84} , transepithelial electrolyte transport studies (using Ussing chambers modified for use with cultured cells), and free cytosolic Ca^{2+} measurements followed procedures similar to those described previously (Dharmasathaphorn *et al.*, 1989b; Dharmasathaphorn & Madara, 1990). Short-circuit current (I_{sc}) was used to quantitate transepithelial Cl^- secretion. Previous studies have demonstrated that T_{84} cells secrete Cl^- in response to carbachol and histamine, and that the resulting changes in I_{sc} are wholly reflective of the amounts of Cl^- secretion induced by these secretagogues (Dharmasathaphorn & Pandol, 1986; Wasserman *et al.*, 1988). In this study, I_{sc} measurements were carried out in Ringer solution containing (in mM): Na^+ 140, K^+ 5.2, Ca^{2+} 1.2, Cl^- 119.8, HCO_3^- 25, $H_2PO_4^-$ 2.4, HPO_4^{2-} 0.4, and glucose 10. Free cytosolic Ca^{2+} measurements had to be carried out in a bathing medium that was relatively low in HCO_3^- to avoid interference caused by precipitation. Therefore, we have modified the Ringer solution used in I_{sc} studies for optimal $[Ca^{2+}]_i$ measurements. This was essentially accomplished by reducing the concentration of HCO_3^- and adding N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid] (HEPES) as a buffer; the solution resembles standard Hanks' buffer. This buffer contained (in mM): Na^+ 141.6, K^+ 5.8, Ca^{2+} 1.2, Mg^{2+} 0.8, Cl^- 144.7, $H_2PO_4^-$ 0.3; SO_4^{2-} 0.8, HCO_3^- 4.1, HEPES 10 and glucose 5.6, with the pH adjusted to 7.4 with NaOH. Preliminary testing indicated that the actions of each secretagogue on I_{sc} in both media were similar (data not shown). Therefore, the I_{sc} measurements which have been obtained with the standard Ringer solution are compared to $[Ca^{2+}]_i$ data obtained with the modified solution. In some experiments, I_{sc} measurements were made in T_{84} monolayers which had been preincubated for 45 min with the acetoxymethyl ester of the intracellular Ca^{2+} chelator, bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (0.15 mM). This concentration of BAPTA is sufficient to abolish completely agonist-stimulated increases in $[Ca^{2+}]_i$ in T_{84} cells (Kachintorn *et al.*, 1992).

Determination of inositol phosphates

T_{84} cells were labelled with *myo*-[2- 3H]-inositol to study the generation of inositol phosphates following stimulation. T_{84} cells were grown to confluency (about 10^6 cells/well) in 6-well

tissue culture plates (35 mm diameter wells). The culture medium was removed and the cells were then washed once with 0.22% trypsin/0.72 mM EDTA solution, which remained on the cells for 5 min. The trypsin/EDTA solution was replaced with 1 ml of inositol-free DMEM/F12 medium (1:1, v/v) supplemented with dialyzed newborn calf serum (5%) and *myo*-[2- 3H]-inositol (12.8 Ci mmol $^{-1}$, 50 μ Ci ml $^{-1}$). Cells were then incubated for 24 h after which time an additional 1 ml of medium containing dialyzed serum was added. The cells were then incubated for another 48 h (72 h total). Control experiments indicated that growth of cells in inositol-free medium did not alter their ability to display ion transport responses (data not shown). After labelling, the medium containing any unincorporated radioisotope was removed and the cells were washed 4 times with Ringer solution containing glucose. The cells were incubated for varying periods of time at 37°C in 500 μ l Ringer solution containing thapsigargin (1 μ M). The incubations were terminated by addition of 1 ml of ice cold methanol and the cells were then sonicated at 4°C until they were detached from the tissue culture plate. The suspension of sonicated cells in methanol was pooled with one methanol wash (in 1 ml) of the well into a polypropylene test tube. Inositol phosphates were extracted by a slight modification of a procedure described by Berridge and co-workers (1983). Briefly, HCl (300 μ l, 0.5 N), 1 mM $CaCl_2$ plus 10 mM mannitol (600 μ l), and chloroform (800 μ l) were added to the extracts and the tubes were then centrifuged. After collecting the upper phase, the interface was washed twice with 1 ml of the upper phase of chloroform/methanol/0.1 M sodium cyclohexane-1,2-diaminetetra-acetic acid (16:8:5 v/v/v). The combined upper phases were then dried and the product was dissolved in 500 μ l of double-

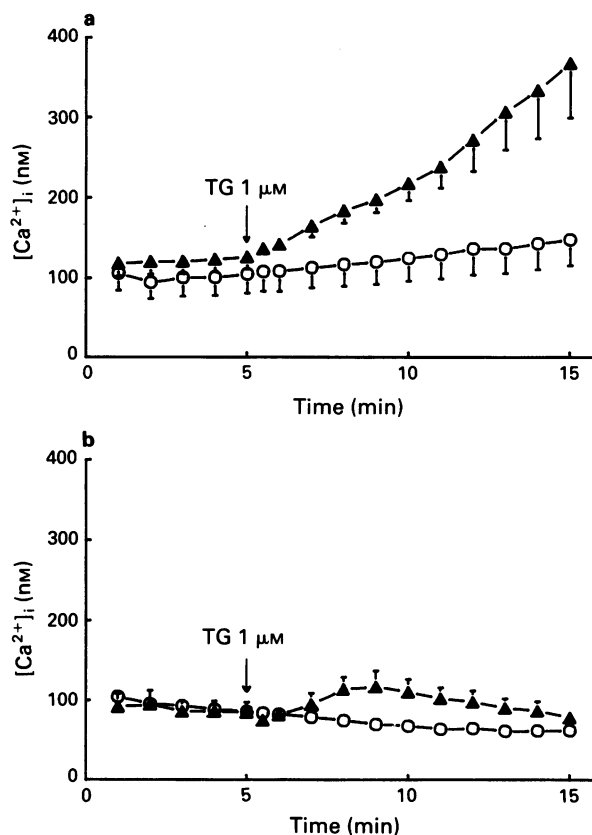


Figure 1 Effect of thapsigargin (TG) on free cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) in T_{84} cell monolayers in the presence (a) or absence (b) of extracellular calcium. In (a) and (b) (O) show the time course of changes in $[Ca^{2+}]_i$ in control monolayers with no additions. In experiments denoted by (▲) TG (1 μ M) was added at 5 min. Values are means \pm s.e.mean for 4 experiments.

distilled water and filtered before being applied to an anion-exchange column.

Inositol phosphates were resolved by anion-exchange high performance liquid chromatography (h.p.l.c.) as described by Auger *et al.* (1989) on a 4.6 × 250 mm Partisil 10 SAX column (Whatman, Clifton, New Jersey, U.S.A.). Elution was performed with a three stage gradient: water for 15 min, followed by a linear gradient to 0.25 M NH₄H₂PO₄ (pH 3.8) over 60 min, followed by a linear gradient to 1 M NH₄H₂PO₄ (pH 3.8) over 50 min, at a flow rate of 1 ml min⁻¹. The column was washed with water (1 ml min⁻¹) for 10 min between application of samples. The radioactivity of the effluent was continuously measured by a 171 radioisotope detector (Beckman, San Ramon, California), which performed automatic peak integration with Beckman System Gold Program software. Identification of the inositol phosphates was based on comparison of their elution times with those of authentic radiolabelled standards (New England Nuclear, Boston, MA, U.S.A.).

Materials

Thapsigargin was kindly provided by Dr O. Thastrup, University Hospital, Copenhagen, Denmark and vasoactive intestinal polypeptide (VIP) was donated by Dr J. Rivier, Salk Institute, La Jolla, California, U.S.A. Carbachol (ICN, Irvine, California, U.S.A.), histamine dihydrochloride (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), fura-2/AM and *bis*-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester (BAPTA/AM) (Calbiochem Biochemical, La Jolla, California, U.S.A.) and *myo*-[2-³H]inositol, (+)-[inositol-2-³H(N)]-inositol 1-phosphate, (+)-[inositol-2-³H(N)]-inositol 1,4-bisphosphate, (+)-[inositol-2-³H(N)]-inositol 1,3,4-trisphosphate, and (+)-[inositol-2-³H(N)]-inositol tetrakisphosphate (New England Nuclear, Boston, Massachusetts, U.S.A.), were purchased from the sources indicated.

Results

Effect of thapsigargin on [Ca²⁺]_i

First, we tested whether thapsigargin had an effect on [Ca²⁺]_i in T₈₄ cells. Figure 1 shows that when cells were bathed in Ca²⁺-containing medium (Figure 1a) the addition of 1 μM

thapsigargin gradually and progressively increased [Ca²⁺]_i. In Ca²⁺-free medium (Figure 1b) the increase in [Ca²⁺]_i caused by thapsigargin was of lesser magnitude and not progressive. Next, we tested whether breakdown of phosphoinositides and the resulting production of inositol phosphates was involved in the Ca²⁺-mobilizing activity of thapsigargin. T₈₄ monolayers prelabelled with [³H]-*myo*-inositol were stimulated with 1 μM thapsigargin and the levels of [³H]-inositol phosphates were analyzed by h.p.l.c. As shown in Figure 2, none of the [³H]-inositol phosphates measured (including inositol 1,4,5-trisphosphate ((1,4,5)IP₃), inositol 1,3,4-trisphosphate, inositol 1,4-bisphosphate, inositol 1,3,4,5-tetrakisphosphate, inositol pentakisphosphate and inositol hexakisphosphate) were increased by thapsigargin. In contrast, we have previously reported that increases in a number of inositol phosphates can be readily demonstrated when T₈₄ cells are treated with the calcium-mobilizing secretagogues histamine or carbachol (Kachintorn *et al.*, 1993).

Effect of thapsigargin on I_{sc}

Having confirmed in the cell type under study that the effects of thapsigargin on [Ca²⁺]_i are not accompanied by the generation of inositol phosphates, we were next able to test whether [Ca²⁺]_i mobilized in the absence of phosphatidylinositol turnover can stimulate chloride secretion. Figure 3 shows that addition of thapsigargin to T₈₄ monolayers mounted in Ussing chambers caused an increase in I_{sc}. The increase in I_{sc} appeared to correlate well with the increase in [Ca²⁺]_i induced by thapsigargin in the presence of extracellular calcium (compare Figure 1a with Figure 3). It should be noted that it is not possible to perform Ussing chamber experiments in the nominal absence of extracellular calcium, so whether a direct correlation exists between the data presented in Figure 1b with changes in I_{sc} cannot be tested. As an alternative strategy, we employed the intracellular calcium chelator, BAPTA. We have previously shown that preloading of T₈₄ monolayers with this agent is able to suppress completely both the increase in [Ca²⁺]_i and I_{sc} induced by carbachol, in a parallel fashion (Kachintorn *et al.*, 1992). As shown in Figure 3, the current experiments revealed that the effect of thapsigargin on I_{sc} was likely to be dependent on its ability to increase intracellular calcium levels. When thapsigargin was added to T₈₄ cell monolayers that had been preloaded with the chelator, no increase in I_{sc} was observed (Figure 3). This inhibition was not the result of non-specific

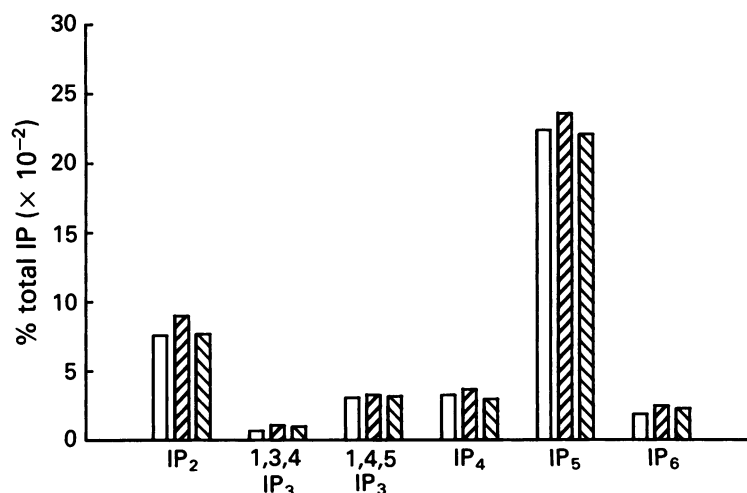


Figure 2 Effect of thapsigargin on levels of ³H-labelled inositol phosphates (IP) in T₈₄ cell monolayers. Levels of IP₂, 1,4,5-IP₃, 1,3,4-IP₃, IP₄, IP₅, and IP₆ are expressed as a percentage of the total counts in inositol phosphates. Standards for IP₅ and IP₆ are not commercially available so their identification is accordingly tentative. Open columns denote levels of each inositol phosphate in control, unstimulated cells. Hatched columns denote levels of each inositol phosphate in cells stimulated with thapsigargin for 1 (▨) or 5 (▩) min. Values are means for 1 experiment performed in duplicate.

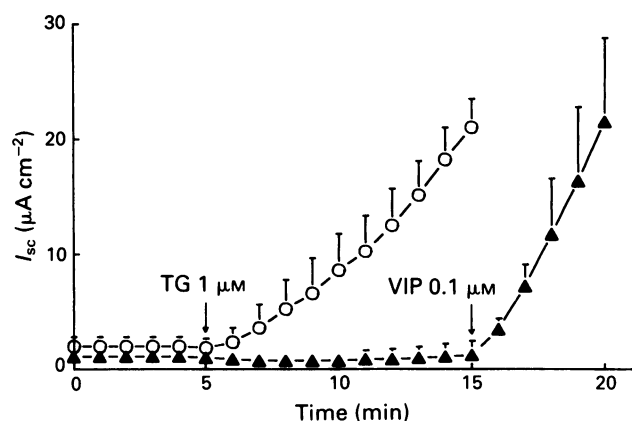


Figure 3 Effect of thapsigargin on chloride secretion (measured as I_{sc}) across T_{84} cell monolayers in the presence (\blacktriangle) or absence (\circ) of BAPTA. Thapsigargin (TG) was added at 5 min. Cells were preincubated with BAPTA, when used, for 45 min prior to mounting. When BAPTA was used, vasoactive intestinal polypeptide (VIP) was added at 15 min to confirm that the chelator did not have non-specific antisecretory effects. Values are means \pm s.e. mean for 4 experiments.

cytotoxicity of BAPTA, since the same cells responded normally to subsequent addition of the cyclic AMP-dependent secretagogue, VIP.

Interaction between thapsigargin and agonists stimulating phospholipid turnover

Carbachol and histamine have been shown to increase $[Ca^{2+}]_i$ in T_{84} and other cell types by an inositol 1,4,5-trisphosphate-dependent mechanism. We therefore added both thapsigargin and carbachol or histamine to T_{84} cells to see if their effects on $[Ca^{2+}]_i$ were additive. Figure 4a shows that the addition of $100 \mu M$ carbachol alone caused an immediate increase in $[Ca^{2+}]_i$ with a slow return to baseline after the first minute. In monolayers where $1 \mu M$ thapsigargin and $100 \mu M$ carbachol were added simultaneously, the initial increase in $[Ca^{2+}]_i$ attributable to carbachol was the same, but subsequently $[Ca^{2+}]_i$ continued to increase. Figure 4b shows similar additive effects when $1 \mu M$ thapsigargin and $100 \mu M$ histamine were added simultaneously. Thus the effects of thapsigargin plus a $(1,4,5)IP_3$ -dependent stimulus on $[Ca^{2+}]_i$ are additive (compare Figures 1 and 4).

Because the Ca^{2+} -releasing effect of thapsigargin was gradual, we attempted to empty the thapsigargin-sensitive Ca^{2+} pool, before the addition of carbachol or histamine, by pretreating T_{84} monolayers with $1 \mu M$ thapsigargin for 10 min. The results are shown in Figures 5 and 6. In either the presence (Figure 5a) or absence (Figure 6a) of extracellular calcium, pretreatment for 10 min with thapsigargin did not significantly alter the change in $[Ca^{2+}]_i$ induced by carbachol. Similarly, the effect of histamine on $[Ca^{2+}]_i$ was not attenuated by thapsigargin in either Ca^{2+} -containing medium (Figure 5) or Ca^{2+} -free medium (Figure 6).

We further investigated a possible relationship between the thapsigargin-sensitive Ca^{2+} pool and the $(1,4,5)IP_3$ -sensitive Ca^{2+} pool by treating cell monolayers with thapsigargin, and then adding carbachol followed by histamine (or *vice versa*). When experiments were carried out in the absence of extracellular Ca^{2+} (Figure 6), pretreatment with thapsigargin prevented the rise in $[Ca^{2+}]_i$ produced by a second stimulation with a $(1,4,5)IP_3$ -mobilizing agonist. Thus when histamine followed carbachol, the small rise in $[Ca^{2+}]_i$ induced by histamine was totally abolished by thapsigargin pretreatment. Similarly, when carbachol followed histamine, carbachol stimulation had no effect on $[Ca^{2+}]_i$ in the presence of thapsigargin.

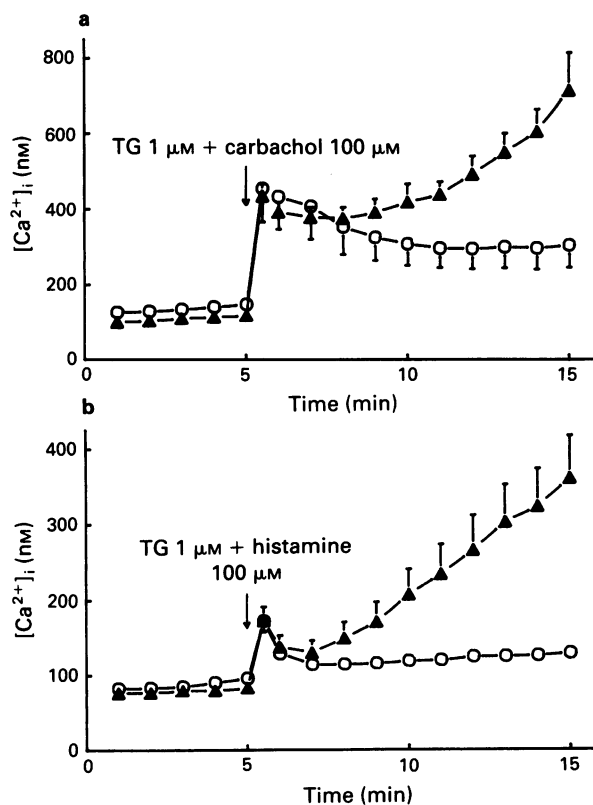


Figure 4 Effect of thapsigargin on $[Ca^{2+}]_i$ in T_{84} cell monolayers when added simultaneously with either carbachol (a) or histamine (b), and in the presence of extracellular calcium: (\circ) changes in $[Ca^{2+}]_i$ when carbachol or histamine were added alone; (\blacktriangle) indicate the time course of changes in $[Ca^{2+}]_i$ following addition of thapsigargin (TG) plus carbachol or histamine at 5 min. Values are means \pm s.e. mean for 4 experiments.

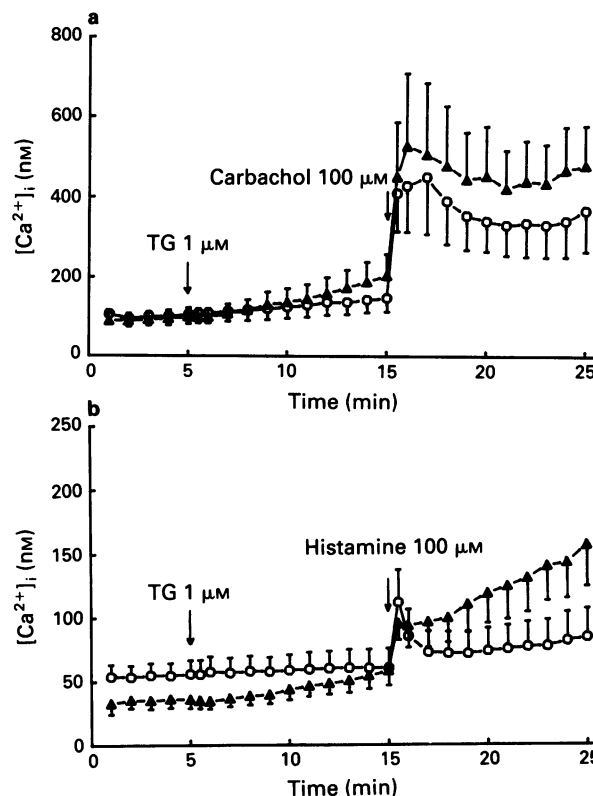


Figure 5 Effects of sequential addition of thapsigargin and either carbachol or histamine on $[Ca^{2+}]_i$ in T_{84} cell monolayers in the presence of extracellular calcium: (\circ) denote results when either carbachol or histamine were added in the absence of thapsigargin (TG); (\blacktriangle) depict experiments where thapsigargin was added at 5 min, followed by carbachol or histamine at 15 min. Values are means \pm s.e. mean for 4–6 experiments.

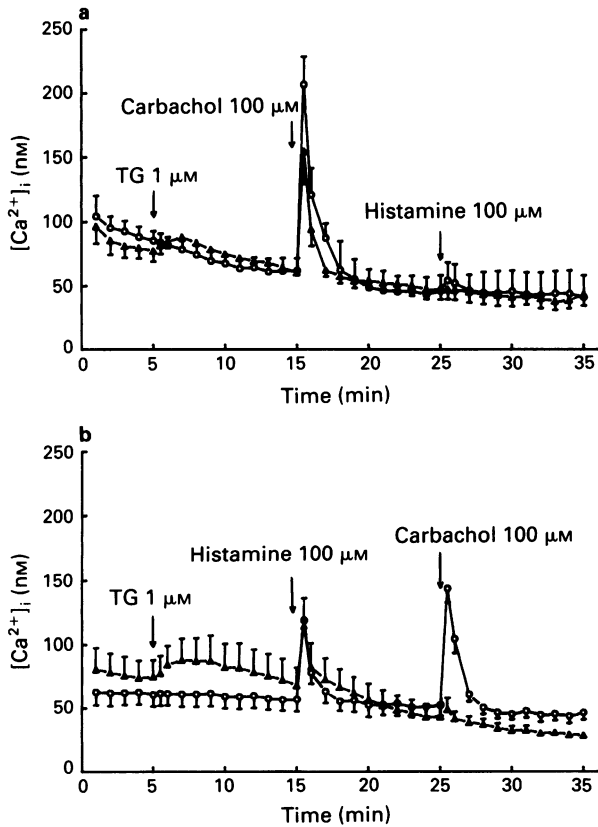


Figure 6 Effect of thapsigargin on $[Ca^{2+}]_i$ responses in T_{84} cells induced by sequential addition of carbachol followed by histamine (a) or histamine followed by carbachol (b) in the absence of extracellular calcium: (○) depict responses in the absence of thapsigargin (TG) while (▲) depict experiments where thapsigargin was added 10 min prior to carbachol or histamine. Values are means \pm s.e.mean for 4 experiments.

The same experimental design was employed in the presence of extracellular calcium to examine possible mechanisms of calcium pool refilling following stimulation. As shown in Figure 7, when calcium was available in the medium, thapsigargin could not prevent a second response to a (1,4,5) IP_3 -dependent stimulus. Thus, when histamine addition was followed 10 min later by carbachol, the latter agent produced essentially equivalent increases in $[Ca^{2+}]_i$ whether or not thapsigargin was present. These findings contrast with the findings of experiments carried out in the absence of extracellular Ca^{2+} (see Figure 6 for comparison). Also of note is the finding that, in cells treated with the sequence of thapsigargin followed by histamine followed by carbachol, $[Ca^{2+}]_i$ still continued to rise significantly compared with cells treated with only the inositol phosphate-dependent stimuli, even after the addition of both carbachol and histamine. This observation suggests that even after treatment with maximal doses of carbachol and histamine, sufficient calcium remains in intracellular stores to be mobilized by thapsigargin and/or the emptying of these stores triggers continued influx of extracellular calcium in the presence of thapsigargin. It was therefore of interest to examine I_{sc} responses when cells were stimulated with sequences of agonists.

As shown in Figure 8, if addition of thapsigargin to the Ussing chamber was followed by either carbachol or histamine addition, the cells were able to display a further increase in I_{sc} which is consistent with the initial spike in $[Ca^{2+}]_i$ that is induced by carbachol or histamine under similar circumstances. This is in keeping with the $[Ca^{2+}]_i$ data shown in Figures 5 and 7. However, pretreatment with thapsigargin appeared to reduce the ability of the cells to display an I_{sc} response to carbachol somewhat, though responsive-

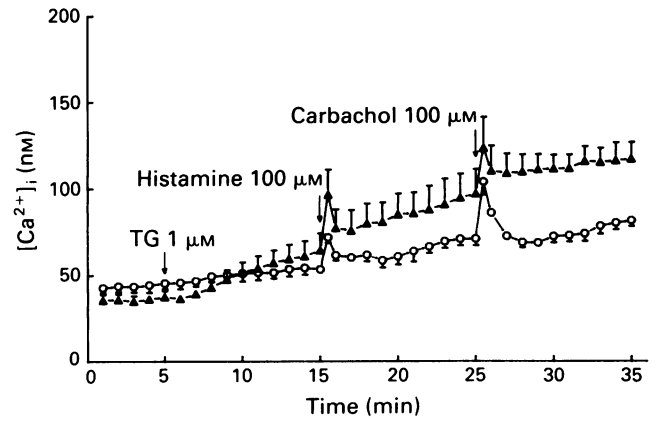


Figure 7 Effect of thapsigargin (TG) on $[Ca^{2+}]_i$ responses in T_{84} cells induced by sequential addition of histamine and carbachol in the presence of extracellular calcium: (○) denote responses in the absence of thapsigargin while (▲) depict experiments where thapsigargin was added at 5 min. Values are mean \pm s.e.mean for 4 experiments.

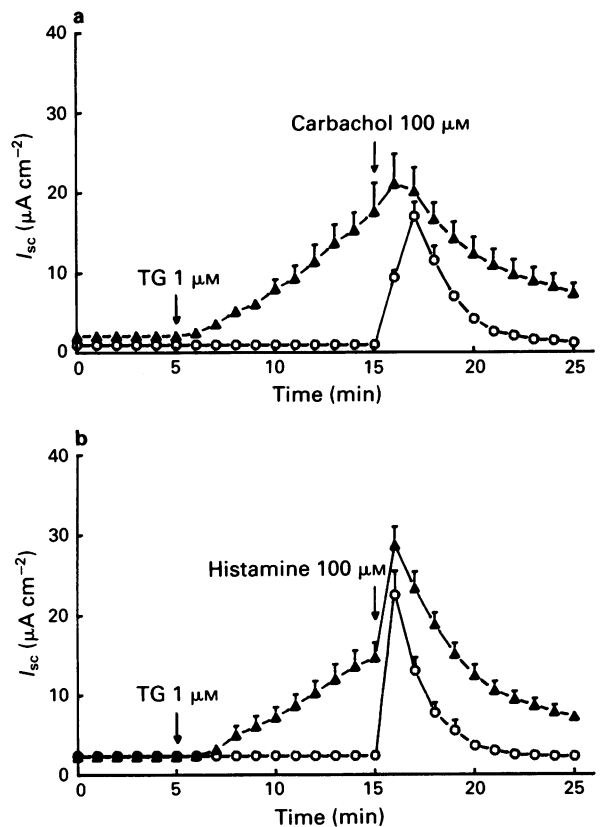


Figure 8 Effect of sequential addition of thapsigargin (TG) and carbachol (a) or histamine (b) on chloride secretion across T_{84} cell monolayers: (○) denote the response to carbachol or histamine alone; (▲) depict experiments where thapsigargin was added at 5 min, which was 10 min before carbachol or histamine addition. Values are means \pm s.e.mean for 5 (carbachol) or 7 (histamine) experiments.

ness to histamine was essentially unaffected. Furthermore, the response to histamine or carbachol was rapidly resolved either in the presence or absence of thapsigargin, even though, in the former case, $[Ca^{2+}]_i$ levels remained elevated (Figure 5).

Discussion

This study was designed to address whether calcium, acting in the absence of any additional second messengers derived

from the turnover of membrane phospholipids, is capable of stimulating transepithelial chloride secretion across a line of human colonic epithelial cells, T₈₄. We had previously demonstrated that such auxiliary second messengers, including diacylglycerol and phosphatidic acid, are likely to contribute to the magnitude of the secretory response stimulated by increases in [Ca²⁺]_i (Kachintorn *et al.*, 1992; Vajanaphanich *et al.*, 1993). In this study, we confirmed that thapsigargin was without effect on the accumulation of any inositol phosphates, and in particular on the levels of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, molecules that are widely recognized as being intracellular mediators of calcium mobilization and/or influx (Hansen *et al.*, 1991; Luckhoff & Clapham, 1992). Thapsigargin was able to increase calcium levels within T₈₄ cells in both the presence and absence of extracellular calcium, though the response was smaller in the nominal extracellular absence of the cation. The smaller response occurring when calcium was not present in the bathing medium may suggest that emptying of an intracellular store directly stimulates calcium influx from the extracellular environment, as has been reported by a number of authors (Takemura *et al.*, 1989; Mason *et al.*, 1991), or might simply reflect the fact that changes in intracellular calcium that are induced by thapsigargin might be more readily dissipated in the absence of extracellular calcium, and therefore less readily detectable via the fura-2 technique.

Overall, qualitatively similar events to those presented here for the effect of thapsigargin on intracellular calcium levels have been described in a number of cell types (Thastrup *et al.*, 1987; 1990; Cheek *et al.*, 1988; Okuchi *et al.*, 1988; Scharff *et al.*, 1988; Benton *et al.*, 1989; Takemura *et al.*, 1989; Foder *et al.*, 1990; Kwan *et al.*, 1990; Law *et al.*, 1990), including another human colonic adenocarcinoma cell line, HCA-7 (Brayden *et al.*, 1989). However, there are some quantitative differences between our findings and those of workers using other cell types. Chief among these was the observation that thapsigargin caused a progressive increase in [Ca²⁺]_i in our cells, whereas in a number of other systems it has been reported to have more rapid and transient effects on [Ca²⁺]_i. This might suggest that the rates of calcium leakage from intracellular stores are different among cell types, or that rates of calcium influx or extrusion across the plasma membrane are likewise variable.

In this study using T₈₄ cells, as well as in most other cell types, a sustained elevation in [Ca²⁺]_i after thapsigargin treatment is dependent on the presence of extracellular Ca²⁺. There is an exception in NG115-401L neuronal cells which only show a transient Ca²⁺ signal (Jackson *et al.*, 1988). Recently, by using extracellular Mn²⁺ as a marker for Ca²⁺-influx induced by thapsigargin, it has been shown that Ca²⁺ entry is responsible for sustaining increased [Ca²⁺]_i (Foder *et al.*, 1990). Furthermore, it has been shown by others that the sustained increase in [Ca²⁺]_i seen with thapsigargin is not due to inhibition of the rate of Ca²⁺ extrusion across the plasma membrane (Takemura *et al.*, 1990). Other inhibitors of endomembrane Ca²⁺-ATPases, such as 2,5-di-(*tert*-butyl)-1,4-hydroquinone (BHQ) may have an additional side effect on plasma membrane calcium permeability, which complicates the interpretation of studies with these drugs (Mason *et al.*, 1991).

Thapsigargin failed to increase inositol phosphate production in T₈₄ cells, confirming previous reports that thapsigargin releases Ca²⁺ from an intracellular store by a mechanism independent of the hydrolysis of phosphoinositides (Jackson *et al.*, 1988; Takemura *et al.*, 1989; Law *et al.*, 1990). In addition Takemura and co-workers (1989) reported that thapsigargin had no effect on levels of phosphatidic acid, a product which results from the degradation of phospholipids other than phosphoinositides by phospholipase C or phospholipase D. Our preliminary data also showed that thapsigargin did not increase phosphatidic acid production in T₈₄ cells (data not shown). Thapsigargin did inhibit resolution of

the increase in [Ca²⁺]_i induced by carbachol and histamine, and therefore most likely inhibits Ca²⁺-recycling into intracellular Ca²⁺ stores, via the endoplasmic reticulum Ca²⁺-ATPase, as reported by others (Thastrup *et al.*, 1990).

Notwithstanding the source of the [Ca²⁺]_i elevation induced by thapsigargin, the ability of this drug to increase *I*_{sc} suggests that calcium alone can be a stimulus of chloride secretion. Thus the primary objective of our study was achieved. Our findings stand in contrast to those of Pickles & Cuthbert (1992) who showed that calcium alone was not sufficient to activate ion transport in a human absorptive epithelium. This discrepancy may reflect differences in the control of absorption vs secretion or tissue-specific mechanisms, and is deserving of additional study. Thapsigargin induced chloride secretion from T₈₄ cells in parallel with its effect on [Ca²⁺]_i. The importance of the mobilized calcium in mediating the observed secretory response was demonstrated with BAPTA; this intracellular chelator abolished the change in *I*_{sc} induced by thapsigargin without affecting the response of the cells to a cyclic AMP-dependent stimulus. Thus calcium is a primary mediator of chloride secretion which is effective in the absence of other messengers generated via phospholipid metabolism. In fact, rather than providing evidence that phospholipid metabolites are required to stimulate chloride secretion, we are led to the opposite conclusion by the observations reported here. The data presented in Figure 8 imply that secretagogues which stimulate both a rise in [Ca²⁺]_i and turnover of phosphatidylinositol induce the formation of auxiliary second messengers which actually uncouple chloride secretion from elevated levels of cytoplasmic calcium. This conclusion is drawn from a comparison of the *I*_{sc} responses shown in Figure 8 with the [Ca²⁺]_i responses depicted in Figures 5 and 7. Similarly, the fall in [Ca²⁺]_i after its elevation by carbachol or histamine is unlikely to be the sole reason for the rapid termination of the Cl⁻ secretory response to these agents. The mechanism of the uncoupling of chloride secretion from [Ca²⁺]_i is the subject of current studies in our laboratory, which have preliminarily implicated inositol tetrakisphosphate as a candidate negative second messenger (Kachintorn *et al.*, 1993).

Our studies also addressed whether there is any correspondence between the intracellular pools of calcium within T₈₄ cells that are mobilized by thapsigargin vs those mobilized by stimulation with carbachol or histamine. Thapsigargin did not abolish an initial [Ca²⁺]_i response induced by these secretagogues in either the presence or absence of extracellular Ca²⁺ (Figures 5 and 6). This might suggest that different pools of calcium are being mobilized by thapsigargin and carbachol or histamine. However, these pools at least appear to be linked in some way because, in the absence of extracellular Ca²⁺, pretreatment with thapsigargin could abolish the histamine-induced [Ca²⁺]_i response following addition of carbachol; similarly, thapsigargin pretreatment abolished the carbachol response following the addition of histamine (Figure 6). In human platelets, the thrombin-sensitive Ca²⁺ pool (a (1,4,5)IP₃-sensitive pool) was not completely included in the thapsigargin-sensitive pool (Thastrup, 1990). The present findings are, however, in contrast to those of other studies showing that thapsigargin and (1,4,5)IP₃ affect the same or substantially overlapping pools of sequestered Ca²⁺, e.g. methacholine as examined in adrenal chromaffin cells and parotid acinar cells, and bradykinin as examined in NG115-401L neuronal cells and chondrocytes (Cheek *et al.*, 1988; Jackson *et al.*, 1988; Benton *et al.*, 1989; Takemura *et al.*, 1989). It is possible that thapsigargin and the IP₃-dependent agonists also access the same calcium store in T₈₄ cells, if the rate of spontaneous leakage from this store is low, so that thapsigargin treatment could only deplete a small fraction of the stored calcium under the conditions studied. This possibility is supported by the data presented in Figure 1, which show that, unlike the effects of thapsigargin in many other cell types, this drug has a progressive effect on [Ca²⁺]_i in T₈₄ cells.

We found that prior release of Ca^{2+} from intracellular stores by thapsigargin and histamine did not affect a subsequent $[\text{Ca}^{2+}]_i$ response induced by carbachol, provided that extracellular Ca^{2+} was present (Figure 6). This contrasts with the findings of experiments carried out in the absence of extracellular Ca^{2+} , when the $[\text{Ca}^{2+}]_i$ response to a second IP_3 -dependent agent was essentially abolished (Figure 5). The results suggest that, in the presence of extracellular Ca^{2+} , depletion of intracellular Ca^{2+} stores by the action of thapsigargin and carbachol or histamine stimulates refilling of Ca^{2+} stores directly, thereby allowing a second $[\text{Ca}^{2+}]_i$ response to carbachol or histamine. This is consistent with the theory that emptying of an intracellular Ca^{2+} pool can serve as a signal for Ca^{2+} entry as described previously (Berridge *et al.*, 1983; Scharff *et al.*, 1988; Takemura *et al.*, 1989). However, it is as yet unclear whether the extracellular Ca^{2+} flows directly into the cytoplasm or via the endoplasmic reticulum. From our observations, it seems reasonable to suggest that Ca^{2+} entry may occur through an endoplasmic reticulum pool before entering the cytoplasm, because the apparent refilling was not accompanied by a change in $[\text{Ca}^{2+}]_i$.

In summary, thapsigargin mobilizes calcium in T_{84} cells, as in other cell types, in a manner that is apparently independ-

ent of phosphatidylinositol breakdown and utilizes both intracellular and extracellular calcium. This increased cytoplasmic calcium can stimulate chloride secretion without a requirement for additional auxiliary second messengers. Indeed, our data suggest that calcium-dependent hormonal secretagogues actually induce the production of inhibitory second messengers which serve to uncouple chloride secretion from calcium elevations. This is in keeping with our current working model of calcium-dependent chloride secretion across T_{84} cells, where events subsequent to, or occurring in parallel with calcium mobilization, such as protein kinase C activation (Kachintorn *et al.*, 1992) or the generation of inositol tetrakisphosphate (Kachintorn *et al.*, 1993) actually serve to limit, rather than promote, the secretory response.

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