CELL REGULATION, Vol. 2, 27–39, January 1991

Thapsigargin potentiates histamine-stimulated HCl secretion in gastric parietal cells but does not mimic cholinergic responses

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The role of calcium in control of HCl secretion by the gastric parietal cell was examined using a recently available intracellular calcium-releasing agent, thapsigargin, which has been shown, in some cell types, to induce sustained elevation of intracellular calcium ($[Ca^{2+}]_i$), an action that appears to be independent of inositol lipid breakdown and protein kinase C activation and to be mediated, at least partially, by selective inhibition of endoplasmic reticulum Ca^{2+}-ATPase. Using the calcium-sensitive fluorescent probe, fura-2, in combination with digitized video image analysis of single cells as well as standard fluorimetric techniques, we found that thapsigargin induced sustained elevation of $[Ca^{2+}]_i$ in single parietal cells and in parietal cells populations. Chelation of medium calcium led to a transient rise and fall in $[Ca^{2+}]_i$, indicating that the sustained elevation in $[Ca^{2+}]_i$ in response to thapsigargin was due to both intracellular calcium release and influx. Although thapsigargin appeared to affect the same calcium pool(s) regulated by the cholinergic agonist, carbachol, and the pattern of thapsigargin-induced increases in $[Ca^{2+}]_i$ were similar to the plateau phase of the cholinergic response, thapsigargin did not induce acid secretory responses of the same magnitude as those initiated by carbachol (28 vs 600% of basal). The protein kinase C activator, 12-O-tetradecanoyl phorbol-13-acetate (TPA) potentiated the secretory response to thapsigargin but this combined response also did not attain the same magnitude as the maximal cholinergic response. In the presence but not the absence of medium calcium, thapsigargin potentiated acid secretory responses to histamine, which elevate both cyclic AMP (cAMP) and $[Ca^{2+}]_i$ in parietal cells, as well as forskolin and cAMP analogues but had no effect on submaximal and an inhibitory effect on maximal cholinergic stimulation. Furthermore, thapsigargin did not fully mimic potentiating interactions between histamine and carbachol, either in magnitude or in the pattern of temporal response. Assuming that the action of thapsigargin is specific for intracellular calcium release mechanisms, these data suggest that 1) sustained influx of calcium is necessary but not sufficient for cholinergic activation of parietal cell HCl secretion and for potentiating interactions between cAMP-dependent agonists and carbachol; 2) mechanisms in addition to elevated $[Ca^{2+}]_i$ and protein kinase C activation may be involved in cholinergic regulation; and 3) increases in $[Ca^{2+}]_i$ in response to histamine are not directly involved in the mechanism of histamine-stimulated secretion.

Introduction

Calcium plays a central role in regulation of stimulus-secretion coupling in a variety of tissues. It is generally accepted that certain classes of hormones, paracrines, neurotransmitters, and growth factors initiate cellular responses through an increase in the cellular messengers, inositol 1,4,5 trisphosphate ($InsP_3$)[1] and diacylglycerol, and subsequent elevation of $[Ca^{2+}]_i$ and activation of protein kinase C. Secretion of HCl by the gastric parietal cell, which lines gastric pits within the gastric mucosa, also appears to depend on changes in $[Ca^{2+}]_i$ and perhaps activation of protein kinase C, at least when stimulated with cholinergic agonists and the hormone, gastrin (Chew, 1989; Forte and Soll, 1989; Soll and Berglindh, 1987). In all, there are three classes of well-characterized stimulants of HCl secretion, including acetylcholine and analogs, gastrin, and cholecystokinin, which appear to act on the same receptor, and histamine, which acts via H_2-type receptors. Both the cholinergic agonist, carbachol, and gastrin have been shown to elevate $[Ca^{2+}]_i$ in isolated parietal cells (Muallem and

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[1] Abbreviations: AP, ^{14}C-aminopyrine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; CPT-cAMP, 8-(-4-chlorophenylthio)adenosine 3':5'-cyclic monophasphate; $InsP_3$, inositol 1,4,5 trisphosphate; TPA, 12-O-tetradecanoyl phorbol-13-acetate.

Sachs, 1985; Chew and Brown, 1986; Negulescu and Machen, 1988), apparently via $InsP_3$-dependent mechanisms (Chew and Brown, 1986), and to activate protein kinase C (Park *et al.,* 1987). Chelation of extracellular calcium has been known for some time to inhibit secretion in response to these agonists (Berglindh *et al.,* 1980; Soll, 1981; Chew, 1985b). In contrast, the stimulatory effects of histamine, which characteristically activate adenylyl cyclase, elevates cyclic AMP (cAMP) (Chew *et al.,* 1980) and activates parietal cell cAMP-dependent protein kinase(s) (Chew, 1985a), are not substantially affected by chelation of extracellular calcium (Berglindh *et al.,* 1980; Soll, 1981; Chew, 1985b). Additional evidence suggesting that histamine initiates HCl secretion via a cAMP-dependent pathway separate from that activated by calcium-dependent agonists includes both in vivo and in vitro observations of potentiating interactions between histamine and carbachol and histamine and gastrin (c.f. Forte and Soll, 1989).

On the basis of such observations, it was generally accepted that histamine exerts its stimulatory effects on HCl secretion via cAMP-dependent pathway and carbachol and gastrin activated other calcium-dependent events, which then initiated HCl secretion. Utilization of calcium-sensitive fluorescent probes such as fura-2 (Grynkiewicz *et al.,* 1985) has indicated, however, that histamine elevates parietal cell $[Ca^{2+}]_i$ by mechanisms involving both intracellular release and extracellular influx (Chew and Brown, 1986; Michaelangeli *et al.,* 1989; Negulescu *et al.,* 1989; Chew and Ljungström, 1990; Ljungström and Chew, 1990). These observations have led to a reinvestigation of the role of calcium in histamine-stimulated HCl secretion. New data have shown that although chelation of medium calcium with ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) has a small to no effect on histamine-stimulated parietal cell acid secretion [measured indirectly in vitro as cellular accumulation of the weak base, ^{14}C-aminopyrine (AP)], chelation of intracellular calcium using the cell-permeant acetoxymethyl ester form of 1,2-bis(2-aminophenoxy)ethane-*N, N, N′, N′*-tetraacetic acid (BAPTA) leads to a partial inhibition of the secretory response. Under the same conditions, secretion in response to carbachol is completely inhibited (Brown and Chew, 1989; Michaelangeli *et al.,* 1989; Negulescu *et al.,* 1989). Since both EGTA and BAPTA may affect cellular events that are not directly related to activation of secretion, these experiments do not conclusively demonstrate a role for calcium in histamine-stimulated secretion.

In recent studies of calcium response patterns in single cultured parietal cells in which secretory responses and changes in $[Ca^{2+}]_i$ can be measured within the same cell (Chew and Ljungström, 1990; Ljungström and Chew, 1990), calcium signaling patterns with histamine were found to be substantially different from those with carbachol. In response to histamine, asynchronous oscillations in the calcium signal were observed in ~50% of cells stimulated with doses of histamine that elicited maximal secretory activity. Oscillations in calcium responses were also observed with submaximal doses of histamine. After cholinergic stimulation, however, oscillations were observed only at doses of carbachol below the EC_{50} for that agonist (Ljungström and Chew, 1990). In addition to differences in calcium signaling patterns, it was observed that although the majority of cells in which histamine-stimulated secretion was detected also responded with increased $[Ca^{2+}]_i$, in ~20% of cells in which secretory responses were observed, there was no detectable change in $[Ca^{2+}]_i$. These data have, therefore, raised additional questions regarding the role of calcium in histamine-stimulated HCl secretion and suggested that different calcium signalling patterns might result from agonist effects on different pool(s) of $[Ca^{2+}]_i$.

Recently, an unusual tumor promoter, thapsigargin, a sesquiterpene lactone occurring naturally in the roots of *Thapsia garganica L.* (Christensen *et al.,* 1982), has been shown to elevate $[Ca^{2+}]_i$ in several different cell types (Thastrup *et al.,* 1987; Ohuchi *et al.,* 1988; Scharff *et al.,* 1988; Brayden *et al.,* 1989; Cheek and Thastrup, 1989; Foder *et al.,* 1989; Takemura *et al.,* 1990; Thastrup *et al.,* 1990). Unlike 12-O-tetradecanoyl phorbol-13-acetate (TPA), thapsigargin has not been found to increase $InsP_3$ or to activate protein kinase C (Hakii *et al.,* 1986; Jackson *et al.,* 1988). With one known exception (NG115-401L cells), thapsigargin produces a sustained rise in $[Ca^{2+}]_i$ that appears to occur via an $InsP_3$-independent mechanism involving specific inhibition of microsomal Ca-ATPase activity (Thastrup *et al.,* 1990) and perhaps calcium influx occurring as a result of depletion of intracellular calcium stores (Thastrup *et al.,* 1990; Putney, 1986). Thus, the mechanism by which thapsigargin elevates $[Ca^{2+}]_i$ presumably involves inhibition of calcium uptake into intracellular pool(s) rather than effects on release mechanisms. In most studies to date, thapsigargin has been utilized to study calcium influx/release mechanisms; however, this agent has been shown to stimulate histamine release by mast cells (Ali *et al.,* 1985; Ohuchi *et al.,*

1989), to cause electrogenic anion secretion in human colonic epithelial cell monolayers (Brayden *et al.,* 1989), to stimulate catecholamine release from adrenal chromaffin cells (Cheek and Thastrup, 1989), to initiate a respiratory burst response in neutrophils and to potentiate the action of TPA-like tumor promoters in these same cells (Kano *et al.,* 1987). Thus, at least in some cell types it appears that sustained elevation of $[Ca^{2+}]_i$ is sufficient to initiate a secretory response.

In this study we utilized thapsigargin as a potential tool to define further the role of calcium in control of stimulus-secretion coupling in the gastric parietal cell in general and, more specifically, the role of calcium in histamine-stimulated secretion. Since initiation of HCl secretion in parietal cells involves a complex series of events, including membrane fusion and relocation of H^+,K^+-ATPase or proton "pump" from intracellular tubulovesicles to the apical or secretory membrane (Forte and Soll, 1989), it was of interest to determine whether or not a simple elevation in $[Ca^{2+}]_i$ was sufficient to initiate a significant secretory response in this cell type.

Results

Effects of thapsigargin on parietal cell $[Ca^{2+}]_i$

Before utilizing thapsigargin to study the role of calcium in HCl secretion, it was necessary to determine 1) whether this tumor promoter elevates $[Ca^{2+}]_i$ in parietal cells and 2) if so, whether the rise in $[Ca^{2+}]_i$ is transient or sustained. In addition, we wished to know if thapsigargin released calcium from the same intracellular pool(s) as carbachol. Both acutely isolated, highly enriched parietal cell populations and parietal cells in primary culture were loaded with the calcium-sensitive fluorescent probe, fura 2, and then were stimulated with thapsigargin at different concentrations (0.1 nM to 1 μM). Initial experiments indicated that thapsigargin induced a maximal rise in $[Ca^{2+}]_i$ between 0.1 and 1 μM with an approximate EC_{50} of 30 nM (not shown). Spectrofluorimetric recordings of responses of highly enriched parietal cell populations (>95%) to 1 μM thapsigargin followed by 100 μM carbachol are shown in Figure 1A. Thapsigargin induced a slow rise in $[Ca^{2+}]_i$ that reached a maximum within ~40 s. Further addition of carbachol did not induce a further rise in $[Ca^{2+}]_i$; however, addition of the calcium ionophore, ionomycin (3 μM) led to a substantial increase in $[Ca^{2+}]_i$ (Figure 1). These data indicate that, unlike thapsigargin, the ionophore affected calcium release/influx mechanisms in addition to those regulated by carbachol. In other experiments (not shown) chelation of medium calcium did not block the initial rise in calcium in response to thapsigargin nor that to secondary addition of ionomycin; however, the rise in calcium was not sustained under these conditions (see single-cell data below). We previously showed that ionomycin transiently elevates $[Ca^{2+}]_i$ in parietal cells upon chelation of extracellular calcium (Brown and Chew, 1989). Present results indicate that thapsigargin also releases calcium from intracellular stores. Since ionomycin caused a further rise in $[Ca^{2+}]_i$ after thapsigargin, which was independent of extracellular calcium, it was concluded that thapsigargin acts more selectively on intracellular calcium pool(s) than calcium ionophores.

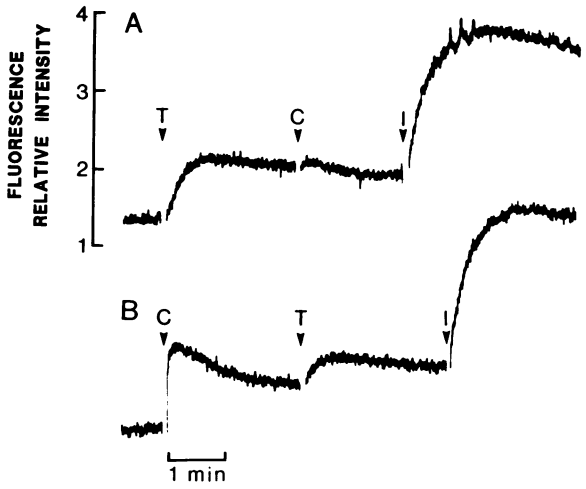

***Figure 1.* Thapsigargin induces sustained elevation of $[Ca^{2+}]_i$ and appears to act on carbachol-sensitive calcium pool(s) in enriched parietal cell populations.** Parietal cells were isolated and enriched using a combination of Nycodenz gradients and centrifugal elutriation then loaded with fura-2 and placed in a thermostated cuvette (37°C) in a spectrofluorimeter as described in Methods. Agonists were added at times indicated in recordings (arrowheads). C, 100 μM carbachol; T, 1 μM thapsigargin; I, 3 μM ionomycin. Recordings in figure were from replicate samplings of the same cell population and were made at 340 nm excitation, 510 nm emission wavelengths. Ratioed 340/380 nm data acquired by rapid manual changes of wavelengths with samples run in parallel yielded the same information (not shown). Experimental protocols were repeated in triplicate in four other cell preparations with similar results.

When cells were first stimulated with carbachol (100 μM) followed by 1 μM thapsigargin, a further rise in $[Ca^{2+}]_i$ was usually detected in cell populations. Ionomycin had a similar effect with this protocol (Figure 1B). Thus, cell population data indicated that thapsigargin and carbachol affected the same calcium pools but suggested that thapsigargin might also release

calcium from carbachol-insensitive pool(s). Since these experiments were performed in cell populations, an alternative explanation was that although not all cells in the population were responsive to carbachol, at least a portion of the carbachol-insensitive cells did respond to thapsigargin. These hypotheses were tested using digitized video image analysis of single cells. Stimulation of single parietal cells with maximal doses of carbachol (100 μM, Figure 2A) elicited a characteristic rapid rise in $[Ca^{2+}]_i$ followed by a decline to a sustained plateau phase. Addition of a maximal dose of thapsigargin after carbachol (1 μM, Figure 2A) elicited no further increase in $[Ca^{2+}]_i$. In the reverse experiment, prestimulation of single cells with 1 μM thapsigargin induced a slow rise in $[Ca^{2+}]_i$ as in cell populations (compare Figures 1A and 2B). Further addition of carbachol (100 μM) caused a small additional increase in $[Ca^{2+}]_i$ in ~28% of cells so challenged (11 of 39 in 9 different preparations). Figure 2B depicts a recording from a cell in which carbachol was found to elicit a small further rise in $[Ca^{2+}]_i$. Based on these single-cell measurements we concluded 1) that thapsigargin affects the same calcium pool(s) as carbachol, although it sometimes less potent and 2) that the calcium response pattern with thapsigargin in single cells mimics the plateau phase of the cholinergic response but not the initial phase which, with carbachol, is characterized by a rapid rise in $[Ca^{2+}]_i$ followed by a relaxation to a lower steady-state level (Muallem and Sachs, 1985; Chew and Brown, 1986; Negulescu and Machen, 1988; Ljungström and Chew, 1990).

In other single cell experiments (Figure 2C), chelation of extracellular calcium with EGTA was found not to prevent the initial thapsigargin-induced rise in $[Ca^{2+}]_i$ but to abolish the sustained phase of the response. These data support conclusions reached from experiments with parietal cell populations (see above) that thapsigargin releases calcium from intracellular pool(s). Thus, the sustained phase of the $[Ca^{2+}]_i$ response to thapsigargin in parietal cells must be due to calcium influx as proposed for hepatocytes (Thastrup *et al.*, 1990) and adrenal chromaffin cells (Cheek and Thastrup, 1989).

Interactions of thapsigargin and TPA on parietal cell acid secretory activity

Thapsigargin alone had only a very small effect on parietal cell acid secretion measured as AP accumulation in isolated, enriched parietal cells and in gastric glands. In parallel experiments, comparisons of thapsigargin-induced AP ac-

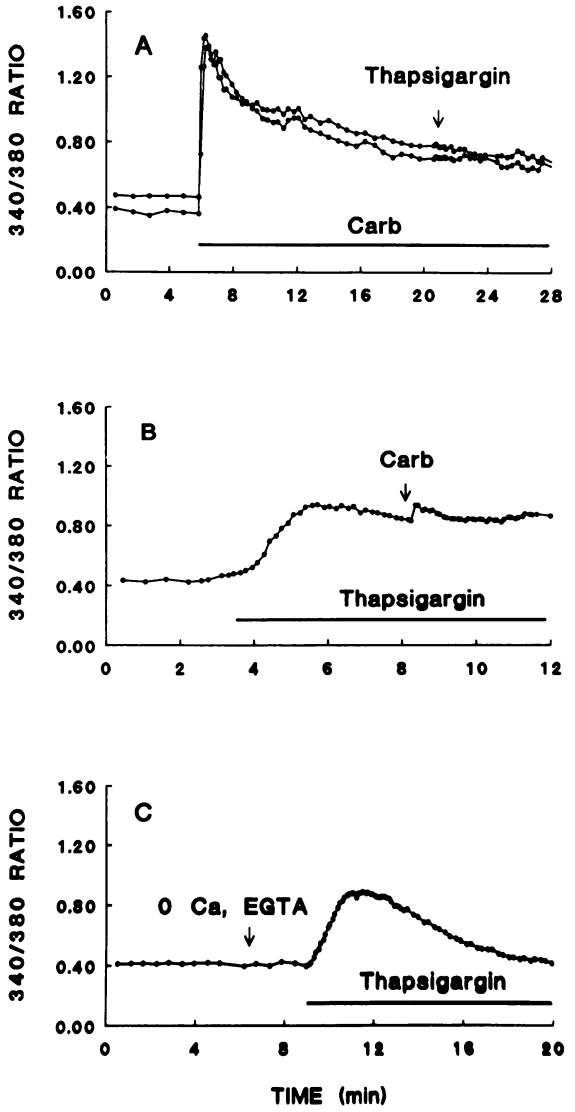

Figure 2. **In single parietal cells in primary culture, prestimulation with carbachol blocks the thapsigargin-induced rise in $[Ca^{2+}]_i$ and pre-stimulation with thapsigargin effectively reduces the cholinergic response.** Cells were isolated, enriched, and placed in primary culture as described in Methods. After 2 d of culture, cells were loaded with fura-2, placed on the stage of a Zeiss IM35 microscope, and images of cellular $[Ca^{2+}]_i$ (detected as changes in fluorescence intensity of intracellular fura 2 with an ISIT camera and expressed as 340/380 nm ratios with correction for background fluorescence) were captured and stored on a SUN 3/280 hard disk using Inovision Ratiotool software routines. Recordings in figure are expressed as 340/380 nm ratios of fura-2 fluorescence instead of $[Ca^{2+}]_i$ because of uncertainties associated with numeric calibration procedures (Ljungström and Chew, 1990). Horizontal lines beneath recordings indicate continued presence of agonist. Thapsigargin, 1 μM; carbachol, 100 μM. Recordings are from two adjacent cells in A and a single cell in B and C. In C, perfusion medium was changed to a nominally calcium-free medium (no added calcium, 0.5 mM EGTA, indicated by arrow in figure) before thapsigargin was added. Data is representative of responses of other cells within the same preparation and of 35 additional cells in 6 other preparations.

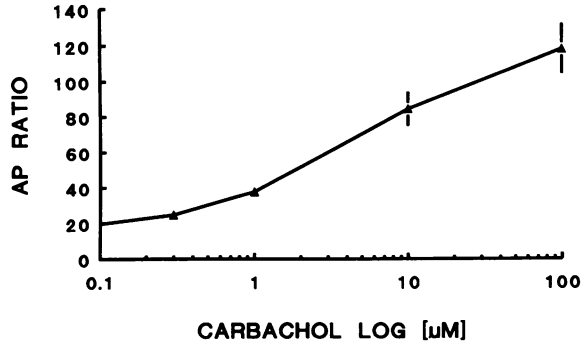

Figure 3. **AP accumulation in gastric glands in response to increasing doses of carbachol.** Gastric glands were isolated by collagenase digestion and ^{14}C-aminopyrine accumulation measured (Methods) after a 45 min temperature equilibration followed by incubation with indicated doses of carbachol for 15 min. Basal AP accumulation ratios averaged 19.7 ± 0.7 (n = 5) for this group of experiments. AP accumulation ratios in response to 0.3 μM carbachol represented minimal detectable carbachol response (25% above basal) and are similar to AP accumulation responses obtained with maximal thapsigargin (see Results).

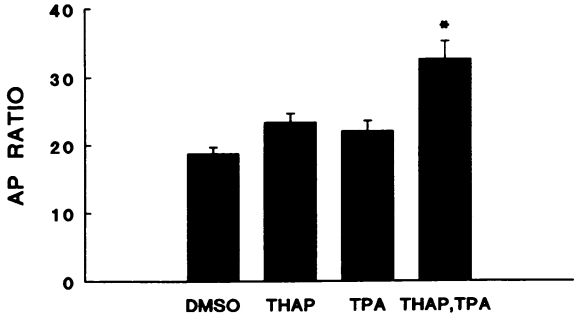

Figure 4. **Combined actions of thapsigargin and TPA elicit a potentiated secretory response in gastric glands.** Glands were isolated and AP accumulation measured as described in Figure 3. Agonists were present in incubation flasks for 15 min. DMSO (0.2%) and cimetidine (10 μM) were added to all flasks; THAP, 1 μM; TPA, 0.1 μM. Values are means ± SEM for nine preparations. *p < 0.01 significantly greater that predicted additive responses.

cumulation with that elicited by different doses of carbachol indicated that the maximal thapsigargin response (28 ± 6% above basal, n = 5) was approximately equivalent to that elicited with a minimally active dose of carbachol (0.3 μM, Figure 3). To test the hypothesis that protein kinase C activation was required to mimic fully the cholinergic response, glands were treated with TPA alone, thapsigargin alone, or thapsigargin + TPA. Maximal doses of TPA were chosen based on previously published data (Brown and Chew, 1987) and from initial experiments in which doses of TPA ranging from 0.1 nM to 1 μM and doses of thapsigargin from 1 nM to 1 μM were tested (not shown). Data in Figure 4 show that the combined effect of TPA + thapsigargin elicited a response that was greater than additive; however, comparison of these data with carbachol dose-response data in Figure 3 indicates that the maximal response that could be elicited with TPA + thapsigargin approximated the response to 1 μM carbachol which is ~4 times less than the EC_{50} (4.5 ± 0.04 μM, n = 5) for that agonist.

Thapsigargin potentiates AP accumulation responses to histamine, forskolin, and cAMP analogues and carbachol

Since thapsigargin produced a rise in $[Ca^{2+}]_i$ similar to the plateau phase elicited by doses of carbachol above the EC_{50} for that agonist (Ljungström and Chew, 1990), it was of interest to determine whether or not this calcium response pattern had any effect on acid secretory-related responses to histamine, which not only to elevates cAMP in parietal cells but also may induce slow, asynchronous oscillations in $[Ca^{2+}]_i$ in this cell type (Chew and Ljungström, 1990; Ljungström and Chew, 1990). With histamine, thapsigargin was found to induce a potentiated response to both low (Figure 5) and maximal (Figure 6) doses of histamine. In dose-response experiments, the potentiating effect of thapsigargin was found to approximate closely the dose range that elicited increases in $[Ca^{2+}]_i$ (Figure 7, EC_{50} = 26 ± 7 nM). In contrast, thapsigargin did not enhance AP accumulation in response to low or high doses of carbachol (Figures 5 and 6) and was found to reduce the response to maximal doses of carbachol (Figure

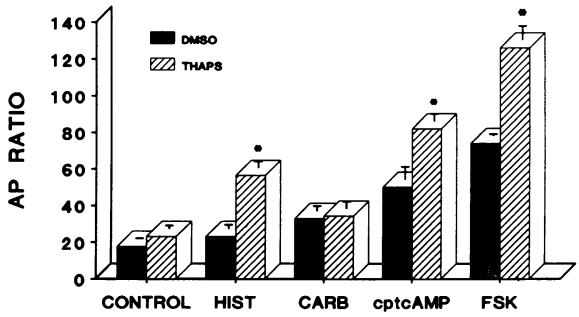

Figure 5. **Thapsigargin potentiates AP accumulation in response to histamine, forskolin, and CPT-cAMP but not carbachol.** Gastric glands were isolated and AP accumulation measurements performed as described in Figure 3. Incubation time with agonists was 10 min. DMSO, 0.1% was included in all flasks. THAPS, 1 μM thapsigargin; HIST, 1 μM histamine; CARB, 0.3 μM carbachol; cptcAMP, 0.5 mM; FSK, 0.3 μM forskolin. Cimetidine (10 μM) was included with all treatments except histamine. Values are means ± SEM for 5–8 experiments. *p < 0.01 significantly different from paired treatment.

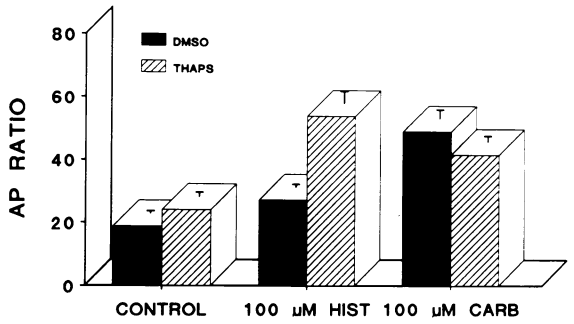

***Figure 6.* Thapsigargin potentiates parietal cell acid secretory activity in the presence of maximal stimulatory doses histamine but not carbachol.** Glands were isolated and AP accumulation measured as described in Figure 3 except incubation time with agonists was 5 min. HIST, histamine; CARB, carbachol; THAPS, 1 μM thapsigargin. DMSO, 0.1%, was included in all flasks. Values are means ± SEM for five experiments.

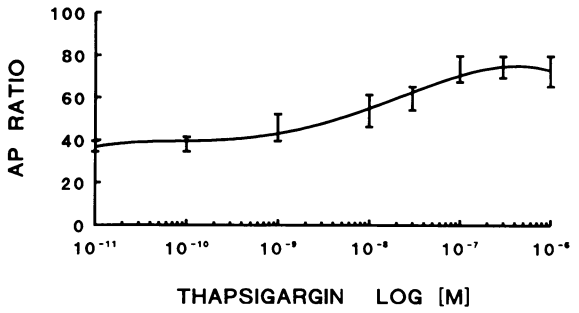

***Figure 7.* Thapsigargin potentiation of histamine-stimulated acid secretory activity is dose-dependent.** Gastric glands were isolated and AP accumulation measured as described in Figure 3. Histamine (1 μM) was present in all flasks. Incubation time with agonists was 10 min. Values are means ± SEM for five experiments. The apparent EC_{50} for the potentiated response was 26 ± 7 nM. Mean AP accumulation ratio with histamine alone was 37 ± 2.5. Basal AP accumulation ratio was 19 ± 1.

6). Since previous data had shown that carbachol, when used at very low concentrations, frequently elicited oscillations in $[Ca^{2+}]_i$ (Ljungström and Chew, 1990), it was of interest to determine if thapsigargin, which causes sustained elevation of parietal cell $[Ca^{2+}]_i$ (Figure 2), would at least produce an additive AP accumulation response under these conditions. In experiments with four different gland preparations, thapsigargin was found not to produce any enhancement of the cholinergic response (AP accumulation ratios were 27 ± 1.8 with 0.3 μM carbachol versus 25 ± 1.7 with carbachol + 1 μM thapsigargin). These data, therefore, suggest that sustained elevation of $[Ca^{2+}]_i$ alone is not sufficient to enhance submaximal cholinergic acid secretory responses. Data in Figure 5 suggest, however, that a sustained rise in $[Ca^{2+}]_i$ is sufficient to potentiate the response to histamine as well as agents that act via cAMP-dependent mechanisms, including the cAMP analogue, 8-(-4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), and forskolin, which activates parietal cell adenylyl cyclase, elevates cAMP, and activates cAMP-dependent protein kinase(s) in parietal cells (Chew, 1989).

Figure 8 shows that chelation of medium calcium totally abolishes the potentiating interaction between histamine and thapsigargin but does not block histamine-stimulated secretion. Similar results were obtained with CPT-cAMP and forskolin (not shown). These data are important because they indicate that 1) a sustained influx of calcium is necessary to elicit a potentiated response with agonists that elevate cellular cAMP and 2) the potentiating effects of thapsigargin are specific with respect to calcium. The lack of effect of thapsigargin on cholinergic responses suggests that the actions of this agent are relatively specific and selective.

Measurement of $[Ca^{2+}]_i$ in single parietal cells showed that when cells were stimulated with histamine and then thapsigargin, a significant further rise in $[Ca^{2+}]_i$ occurred (Figure 9). These data contrast with those in Figure 2A in which thapsigargin did not induce a further rise in $[Ca^{2+}]_i$ after maximal stimulation with carbachol. Thus, thapsigargin appears to act on intracellular calcium pool(s) different from, or in addition to, those affected by histamine. Limitations in resolution of our video image analysis system presently preclude precise cellular localization of these putative calcium pools.

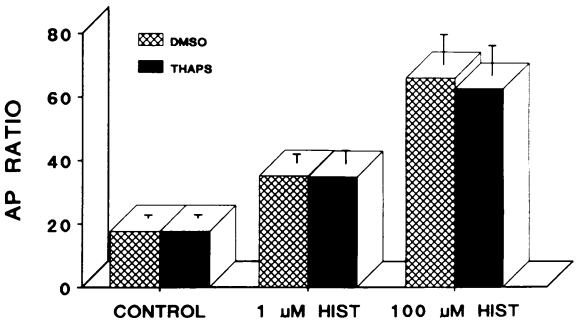

***Figure 8.* Chelation of extracellular calcium completely blocks potentiating interactions between thapsigargin and histamine.** Glands were isolated and AP accumulation measured as in Figure 3 except that before pre-incubation, glands were rinsed in nominally calcium-free incubation media (0.5 mM EGTA, no added Ca^{2+}) and incubated in this same media. After a 30-min temperature equilibration, incubation time with agonists was 10 min. DMSO (0.1%) was included in all flasks; HIST, histamine; THAPS, thapsigargin (1 μM). Values are means ± SEM for six experiments.

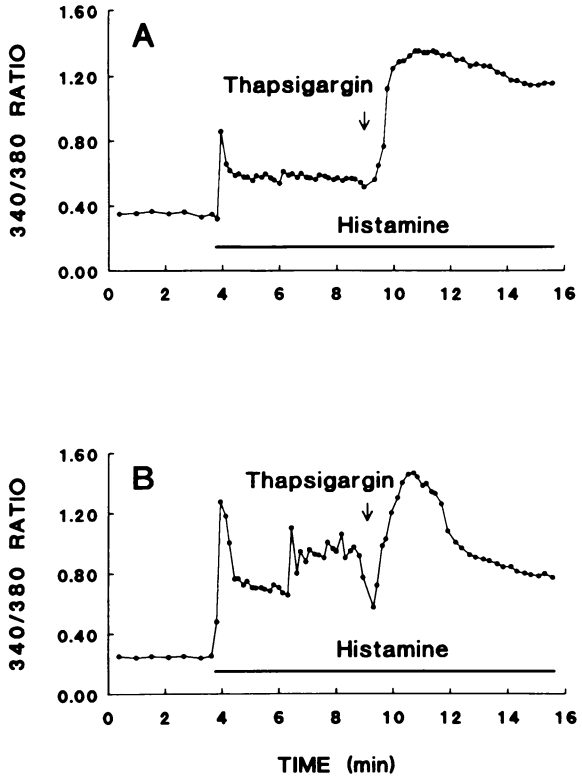

Figure 9. **Thapsigargin induces a rise in $[Ca^{2+}]_i$ following stimulation with maximal doses of histamine.** Addition of histamine (100 μM) to responding parietal cells in culture resulted in either an initial spike followed by a rapid decline to near base-line levels (A) or repeated oscillations in $[Ca^{2+}]_i$ [~50% of cells sampled (B, see also Ljungström and Chew, 1990)]. With both response patterns, thapsigargin (1 μM) further elevated $[Ca^{2+}]_i$ and oscillations disappeared. It is not yet clear whether the disappearance of oscillations is due to an effect of thapsigargin on an overlapping, histamine-sensitive pool or a masking of the oscillations due to an action of thapsigargin on other intracellular calcium pool(s). As shown in the figure, in some cases the thapsigargin-induced rise in $[Ca^{2+}]_i$ declined somewhat following the initial rise. This decline occurred in some, but not all cells, independent of the initial histamine response pattern and was also occasionally observed with thapsigargin alone. In these experiments, cells were loaded with fura-2 and changes in 340/380 ratios measured as described in Figure 2. Recordings are from two adjacent cells. Data are representative of 65 cells from 6 different preparations in which 55 cells (85%) responded initially to histamine and 64 responded to further addition of thapsigargin (98%).

Comparison of potentiating interactions between histamine and carbachol and histamine and thapsigargin

Demonstration that a sustained elevation in $[Ca^{2+}]_i$ can potentiate AP accumulation in response to histamine suggested that elevated calcium alone might be sufficient to mimic the potentiating interaction between carbachol and histamine. To test this hypothesis, parallel time-course experiments were performed with carbachol + histamine and thapsigargin + histamine. We chose a dose of carbachol (0.3 μM) that matched, as closely as possible, the AP accumulation response to a maximal dose of thapsigargin (0.5 μM). Comparison of time course data obtained under these conditions indicated that thapsigargin did not completely mimic the effects of carbachol. With thapsigargin + histamine (1 μM), the potentiating interaction was not sustained as was the interaction between carbachol and histamine and was of lesser magnitude than that in response to carbachol + histamine (compare Figures 10 and 11). These data suggest that although calcium can partially mimic the potentiating interaction between histamine and carbachol, other factors

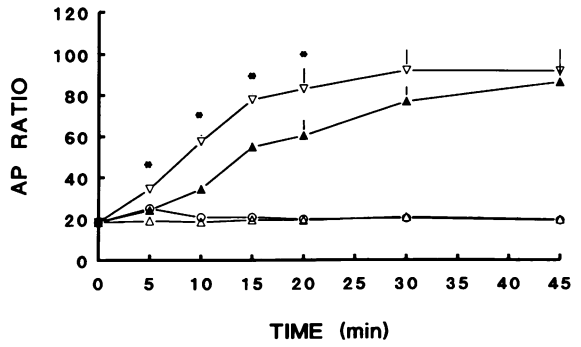

Figure 10. **Time course of potentiating interactions between thapsigargin and histamine.** Glands were isolated and AP accumulation measured as in Figure 3. △, basal; ○, 1 μM thapsigargin; ▲, 1 μM histamine; ▽, 1 μM histamine + 1 μM thapsigargin. DMSO (0.1%) was included in all flasks. Values are means ± SEM for six experiments. *$p < 0.01$ significantly different from additive response of histamine plus thapsigargin.

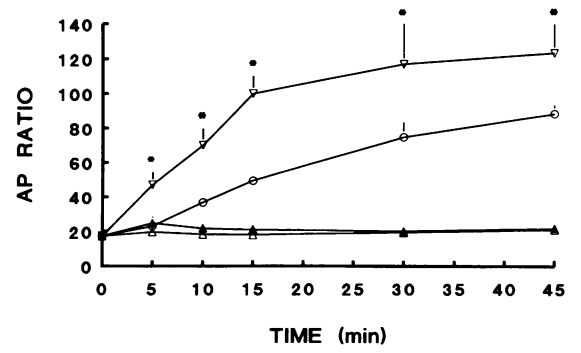

Figure 11. **Time course of potentiating interactions between histamine and carbachol.** Glands were treated as described in Figure 9 except carbachol was used in place of thapsigargin. △, basal; ▲, 0.3 μM carbachol; ○, 1 μM histamine; ▽, 0.3 μM carbachol + 1 μM histamine. Values are means ± SEM for five experiments. *$p < 0.01$ significantly different from additive response of histamine plus carbachol.

may also play a role in the interaction. Attempts to mimic the complete response with thapsigargin (0.5 μM) + TPA (10 nM) + histamine (1 μM) were unsuccessful in that further addition of TPA reduced rather than enhanced the potentiating interaction between histamine and thapsigargin (not shown).

Discussion

In this study we have used a recently available non-phorbol ester, calcium-elevating tumor promoter, thapsigargin, to provide evidence that, in contrast to a number of other cell types, a simple rise in $[Ca^{2+}]_i$ is not a sufficient stimulus to initiate significant HCl secretion in isolated parietal cells. The weak effect of thapsigargin on AP is not entirely unexpected in that stimulus-secretion coupling in the gastric parietal cell is considerably more complicated than, e.g., fixed ion transport processes or simple exocytosis, both of which have been shown to be activated by thapsigargin (Ali *et al.,* 1985; Brayden *et al.,* 1989; Cheek and Thastrup, 1989; Ohuchi *et al.,* 1989). In contrast to these morphologically simpler cell types, the unstimulated gastric parietal cell possesses an extensive intracellular membranous network that includes internal tubulovesicular membranes containing gastric H^+,K^+-ATPase (the proton "pump") and intracellular membranous canaliculi with protruding short microvilli. After agonist stimulation, tubulovesicular membranes fuse with the apical, canalicular membrane inserting proton pumps into this membrane and dramatically extending the length and number of microvilli. During this process a neutral KCl-conductive pathway (Forte and Soll, 1989) or a Cl^- conductance alone (Demarest and Loo, 1990) have been reported to be inserted into the apical canalicular membrane.

The internal complexity of the parietal cell is also externalized in that this cell type is modulated by neural as well as paracrine and endocrine factors secreted by other cell types within the gastric mucosa; hence, the study of second-messenger control mechanisms in these cells is best performed in isolated cell models in which the cellular environment can be more readily controlled. One difficulty with utilizing isolated cells is that HCl secretion must be measured indirectly using intracellular accumulation of weak bases as in this study and/or oxygen consumption as indexes of secretory activity. Both secretory indexes have been well characterized in the literature (Soll and Berglindh, 1987; Forte and Soll, 1989); however, not all test agents can be successfully utilized with these indirect measurements because some exert nonspecific effects either on weak base accumulation or on respiration (Chew, 1989). Past attempts to demonstrate a role for calcium in parietal HCl secretion through the use of the calcium ionophores have been controversial (Chew, 1985b; Michaelangeli *et al.,* 1989), possibly because these ionophores not only elevate $[Ca^{2+}]_i$ but also interfere with AP accumulation, perhaps by activating a Ca^{2+}/H^+ exchanger in these cells (Chew, 1989). When used in chambered mucosae in which apical and basolateral membranes can be maintained separately and HCl secretion can be measured directly, calcium ionophores have been reported to stimulate HCl secretion (Jiron *et al.,* 1981); however, it was not clearly demonstrated that secretion so stimulated was due to direct effects of ionophores on parietal cells rather than release of endogenous stimulatory factors.

Tests of thapsigargin as a potential probe to determine the relative importance of calcium in parietal cell secretion suggested that this agent is considerably more selective than calcium ionophores both in terms of secretory and calcium-releasing mechanisms. As in several other secretory cells (Thastrup *et al.,* 1990), thapsigargin induced a sustained elevation in parietal cell $[Ca^{2+}]_i$ that appeared to result from activation of both intracellular release and extracellular influx mechanisms. If the action of thapsigargin is indeed specific for the Ca^{2+}-ATPase associated with hormonally regulated intracellular calcium pool(s), however, calcium must be recycled rather rapidly between the cytosol to these pool(s) because the maximal calcium response to thapsigargin occurs within 1 min of addition. Considering the rapidity of the rise in $[Ca^{2+}]_i$ in response to thapsigargin, the assumption that this agent acts solely as an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase should be considered carefully since such a rapid recycling of calcium in selected intracellular pools seems unlikely. With respect to observed effects of thapsigargin on parietal cell $[Ca^{2+}]_i$, a similar response pattern has been observed in hepatocytes and adrenal chromaffin cells (Cheek and Thastrup, 1990; Thastrup *et al.,* 1990) but not in the cloned mammalian neural cell line, NG115-401L (Thastrup *et al.,* 1989), suggesting that calcium release/influx mechanisms may be similar in some but not all cell types. Since maximal doses of thapsigargin suppress further carbachol-induced increases in $[Ca^{2+}]_i$, it appears that thapsigargin affects the same calcium pool(s) as carbachol. Recent studies with rat hepatocytes (Thastrup *et al.,* 1990) suggest that at least two pharmacologi-

cally distinct calcium pools, one $InsP_3$-sensitive and one GTP-sensitive, may be affected by thapsigargin. Whether or not similar events occur in parietal cells remains to be determined.

In contrast to thapsigargin, the calcium ionophore, ionomycin, induced a substantial rise in parietal cell $[Ca^{2+}]_i$ when added after a maximal dose of thapsigargin, supporting previous proposals that this ionophore elevates $[Ca^{2+}]_i$ by a mechanism different from thapsigargin (Ali *et al.,* 1985). Unlike calcium ionophores (Chew, 1985b and Chew, unpublished observations), thapsigargin was found not to inhibit AP accumulation in response to histamine, forskolin, and cAMP analogues and indeed potentiated the responses to these agents. Moreover, chelation of medium calcium, which has been shown to have little effect on histamine-stimulated secretion in gastric glands (Berglindh *et al.,* 1980; Chew, 1985b), completely inhibited thapsigargin potentiation of histamine responses, indicating that the potentiation was indeed due to a sustained elevation of $[Ca^{2+}]_i$ induced by thapsigargin and not some other action of this agent. Thus, although we cannot state unequivocally that thapsigargin has no action other than inhibition of reuptake of calcium into agonist-sensitive intracellular calcium pool(s) (Thastrup *et al.,* 1990), this agent did not appear to have detectable nonspecific inhibitory effects on parietal cell acid secretory activity measured as AP accumulation. If, e.g., thapsigargin had a protonophoric activity, as appears to be the case with ionomycin, or nonspecifically inhibited H^+,K^+-ATPase activity, basal and agonist-stimulated AP accumulation should have been reduced upon addition of this agent, and such was not the case.

Since thapsigargin appeared to be selective for carbachol-sensitive pools and calcium signaling patterns have been found to differ with histamine and carbachol, it was important to assess the pattern of the calcium response to thapsigargin in single cells; i.e., did this agent induce oscillations in calcium signaling patterns as had been observed with histamine and low doses of carbachol or was the calcium response sustained? Also, what effect did thapsigargin have on histamine-stimulated increases in $[Ca^{2+}]_i$? Recordings from single parietal cells indicated that thapsigargin induced a sustained, almost monophasic elevation in $[Ca^{2+}]_i$ not unlike the sustained phase of the cholinergic response. Despite the similarities in the calcium signaling patterns, however, thapsigargin did not mimic cholinergic stimulation of HCl secretion. We have interpreted these data as indicative of a requirement for activation of intracellular events in addition to a rise in $[Ca^{2+}]_i$. These observations combined with previous data showing complete inhibition of the cholinergic response with intracellular calcium chelation (Brown and Chew, 1989; Michaelangeli *et al.,* 1989; Negulescu *et al.,* 1989) indicate that calcium is necessary but not sufficient for initiation of HCl secretion. The observations that thapsigargin potentiates AP accumulation stimulated not only by forskolin and cAMP analogs but also by histamine suggests that histamine-induced increases in $[Ca^{2+}]_i$ are not associated with control of HCl secretion. If they were, one would not expect to be able to potentiate histamine-stimulated AP accumulation by elevating $[Ca^{2+}]_i$. Since thapsigargin induced a further rise in $[Ca^{2+}]_i$ after addition of maximally stimulatory doses of histamine, it appears that histamine acts on a different calcium pool as has been observed with different agonist classes in adrenal chromaffin cells (O'Sullivan *et al.,* 1989) or on a shared pool that is not involved in regulation of HCl secretion. We propose, therefore, that histamine-stimulated increases in $[Ca^{2+}]_i$ may be associated with as yet unknown cellular activities and not HCl secretion per se. Our unique findings that thapsigargin potentiates both histamine and cAMP-dependent acid secretory responses in parietal cells not only provide additional support for a relatively selective action of thapsigargin but also indicate that a sustained elevation in $[Ca^{2+}]_i$ can mimic, at least partially, potentiating interactions between histamine and carbachol. As with all pharmacologic agents, however, it would be unwise to assume that the actions of thapsigargin are completely specific for intracellular calcium-uptake mechanisms.

Present data also do not conclusively demonstrate whether or not other factors in addition to elevated $[Ca^{2+}]_i$ are necessary in potentiating interactions between histamine and carbachol. It is possible, e.g., that there are subtle differences between calcium signaling patterns with thapsigargin versus carbachol that are important in eliciting a maximal and sustained potentiation of histamine-stimulated secretion. Another possibility is that activation of protein kinase C or some other intracellular event is required for complete potentiation. As for a number of other secretory cell types, a role for protein kinase C in initiation of parietal cell HCl secretion has been considered by a number of researchers. Earlier studies have shown that carbachol activates protein kinase C in parietal cell-enriched fractions (Park *et al.,* 1987). The protein kinase C activator, TPA, also activates protein kinase C in parietal cell fractions (Chew,

1985a) and increases phosphorylation of at least three parietal cell proteins that are phosphorylated in response to carbachol (Brown and Chew, 1989). Unfortunately, the effects of TPA on parietal cell function are known to be complex, in that TPA stimulates oxygen consumption, an indirect measure of secretory activity, but has little to no effect on basal AP accumulation and a time-dependent inhibitory effect on histamine-stimulated AP accumulation (Anderson and Hanson, 1984; Muallem *et al.,* 1986; Brown and Chew, 1987; Chew, 1989). As discussed previously (Brown and Chew, 1987), the inhibitory action of TPA appears to be associated with initial activation events, perhaps at the level of the receptor or upon hormone-sensitive G protein(s). Others (Anderson and Hanson, 1984) have suggested a more distal effect. Since protein kinase C has been implicated in cholinergic response mechanisms and we believe the inhibitory effect to be at or near the receptor, it was reasonable to determine whether or not co-addition of TPA and thapsigargin would mimic the cholinergic response under conditions where agonist-receptor interactions were bypassed. Although a potentiated response did result when these two agents were added together, the overall response was substantially less than a maximal cholinergic response. Furthermore, addition of TPA along with histamine and thapsigargin not unexpectedly, based on previous results, reduced AP accumulation as compared with that with histamine plus thapsigargin. Thus, present data support a potential stimulatory role for protein kinase C in cholinergic activation of HCl secretion but also emphasize the complex actions of this agent. These data further suggest that the inhibitory action of TPA is at a site proximal to that affected by elevated $[Ca^{2+}]_i$ in conjunction with activation of protein kinase C; otherwise no potentiating interaction between these agents would have been detected. Precise localization of the site at which TPA exerts its inhibitory and excitatory effects remains to be determined. The inability of TPA 1) to induce a potentiation of thapsigargin-stimulated secretion of the same magnitude as the carbachol response and 2) to enhance further the potentiating interaction between histamine and carbachol could also be due to differential activation of protein kinase C isotypes (Parker *et al.,* 1989; Noar, 1990). The ability to discriminate among these various possibilities will require the development/isolation of more selective activators of the different isotypes.

A potentially important observation is that despite the apparent selective action of thapsigargin on carbachol-sensitive calcium pool(s), thapsigargin did not enhance carbachol-stimulated secretion even when minimally stimulating doses of carbachol were utilized. In an earlier study (Brayden *et al.,* 1989), thapsigargin was found to attenuate electrogenic anion secretion in colonic epithelial cells when added after high doses of A23187 or lysylbradykinin, which acts via calcium-dependent mechanisms in this cell type; however, unlike the present study, lower doses of these agents were not tested. The lack of additivity at low doses and inhibition at higher doses of agonists that elevate $[Ca^{2+}]_i$ further supports our conclusion that a sustained rise in $[Ca^{2+}]_i$ is not sufficient in and of itself to elicit a secretory response.

In summary, present results suggest that thapsigargin is a potentially useful probe for the study of the role of calcium in control of parietal cell HCl secretion. Data acquired with thapsigargin thus far indicate calcium to be important in initiating potentiating interactions between cAMP- and calcium-dependent agonists but to be insufficient in and of itself to elicit significant acid secretory activity or to sustain potentiating interactions between different classes of agonists. Further studies are needed to define precisely intracellular events modulated by thapsigargin, particularly with respect to its specificity for intracellular calcium release, and to compare these processes with those controlled by cholinergic agonists to determine whether or not these agents affect similar intracellular events. It is expected that carbachol will be found to regulate processes in addition to those affected by thapsigargin; however, thapsigargin may well prove to be an important tool for dissection of calcium-dependent versus calcium-independent intracellular events.

Methods

Cell isolation and culture

Parietal cells and gastric glands (small groups of parietal and chief cells) were isolated from gastric mucosae of male, 2–3 kg New Zealand White rabbits as previously described (Chew and Brown, 1986). In brief, the mucosa was perfused, under high pressure, in situ with phosphate-buffered saline and then digested with either collagenase (Type II, 50 mg/ml, Sigma Chemical Co., St. Louis, MO) to produce glands or briefly with pronase (20 mg/ml, Calbiochem Corp., San Diego, CA) followed by collagenase (40 mg/ml) to produce single cells. Enriched fractions of parietal cells were isolated with Nycodenz (Accurate Chemical, Westbury, NY) density gradients followed by centrifugal elutriation (JE 5.0 elutriator rotor and J6M centrifuge; Beckman, Fullerton, CA).

For primary culture, parietal cells were collected under sterile conditions from the elutriator rotor, washed several times by low speed centrifugation in culture medium containing antibiotics without bicarbonate (1:1 Ham's F12/Dulbecco's Modified Eagle's medium), and then preattached

in flasks coated with 5% fetal calf serum in the same medium plus amphotericin (Chew *et al.,* 1989). Following preattachment, cells were plated onto dried Matrigel (1:7 dilution, Matrigel/sterile H_2O) and allowed to attach overnight in a 37°C humidified air incubator. Cells were used after 1–3 d of culture.

Cellular incubations for all procedures were performed using a basic medium containing the following components expressed in millimoles per liter: NaCl, 114; KCl, 5.4; Na_2HPO_4, 5.0; NaH_2PO_4, 1.0; $MgSO_4$, 1.2; $CaCl_2$, 1.0; dithiothreitol, 0.5; pyruvate, 1.0; glucose, 10; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 10, pH 7.4 plus bovine serum albumin, fraction V (2 mg/ml); and phenol red (10 μg/ml).

Measurement of acid secretory responses

Parietal cell HCl secretion cannot be measured directly in isolated parietal cells or glands. This is because, unlike the in vivo situation in which HCl is secreted at the apical membrane into the gastric lumen, in acutely isolated and cultured parietal cells, the apical membrane becomes sealed off from the extracellular medium and acidic secretions accumulate within vacuoles inside the cells (Chew *et al.,* 1989; Ljungström and Chew, 1990). In gastric glands the situation is slightly different in that acidic secretions accumulate initially within parietal cells then move into the gland lumen which is sealed off from the extracellular medium during isolation (Dibona *et al.,* 1979). Fortunately, indirect measurement of acid secretion using the weak base, AP, has been shown in numerous studies to provide an excellent measure of secretory responsiveness (c.f. Forte and Soll, 1989; Chew, 1989). In the present study, AP accumulation was measured using modifications of the original Bergdlindh technique (Bergdlindh and Öbrink, 1976) as previously described (Chew and Hersey, 1982; Chew *et al.,* 1989). Glands were used most often because they can be isolated in larger quantities and have preserved apical membrane polarity. Major groups of experimental results were also confirmed in at least two additional experiments with enriched parietal cells. Cells or glands were preincubated with 0.1 μCi/ml AP (specific activity 60–120 mCi/ml; New England Nuclear, Boston, MA), 30–60 min, 37°C in a metabolic shaking incubator prior to agonist addition. With all agonists except histamine, the histamine H_2-receptor blocker, cimetidine (10 μM) was included in the medium to avoid potential problems with agonist-induced histamine release (Nylander *et al.,* 1985). In experiments with thapsigargin and TPA, dimethylsulfoxide (DMSO) was added as a vehicle in equal concentrations in all flasks. DMSO concentrations never exceeded 0.2% and were 0.1% in most experiments. Incubation times for potentiation experiments were chosen based on preliminary time course experiments in which chosen times were found to yield maximal potentiation. Experiments were terminated by withdrawal of aliquots of cells/glands from incubation flasks, rapid centrifugation, and rinsing with incubation medium that did not contain AP. Aliquots of cellular supernatants and dissolved pellets were counted in a Beckman scintillation counter (LS6800) with disintegrations per minute (DPM) correction. AP accumulation values were expressed as the ratio of cellular DPM to supernatant DPM with correction for trapped counts using sodium thiocyanate (10 mM) that completely abolishes cellular AP accumulation (Chew and Hersey, 1982).

Measurement of $[Ca^{2+}]_i$ with fura-2

Intracellular calcium concentrations were estimated in acutely isolated parietal cell populations and in single cultured parietal cells according to previously published techniques (Chew and Brown, 1986; Ljungström and Chew, 1990). Cells were loaded with the fluorescent calcium indicator, fura-2 (Grynkiewicz *et al.,* 1985), using the cell permeant acetoxymethylester (AM) form (4 μM, 20–30 min, 37°C). In cell population experiments, cells were rinsed via brief centrifugation and placed in a thermostated (37°C), stirred cuvette in a spectrofluorimeter (650-40; Perkin Elmer). To acquire ratioed values, cells were alternately excited at 340 and 380 nm [slit widths 5.5 (excitation) and 10 nm (emission)] with the emission wavelength set at 510 nm. Signal calibration and corrections for autofluorescence and leakage of fura into the medium were performed as described by Chew and Brown (1986).

Single cell measurements utilized cells grown on Matrigel-coated, glass-bottomed dishes. Cells were placed on the heated stage of an inverted microscope (IM-35; Zeiss, Thornwood, NY) equipped with quartz condenser and nosepiece and 100× objective (UV-Flor; Nikon, Garden City, NY) and perifused with warm (37°C) medium. Alternate 340/380 nm illumination using 10-nm bandwidth filters in conjunction with neutral density filters (85–97% light attenuation; Omega Optical, Brattleboro, VT) was performed using a computer-controlled electronic shutter (Vincent Associates, Rochester, NY) in combination with a xenon lamp and six-place filter wheel (LEP Inc., Hawthorne, NY). A 405-nm dichroic mirror was used to deflect light through the objective. Fluorescence emission was collected through a 510-nm bandpass filter with an ISIT video camera (model 66 DAGE) which was interfaced to a SUN 3/280 minicomputer via hardware/software from Inovision Corporation (Raleigh, NC). Eight- to thirty-two-frame averages were collected at each excitation wavelength every 5–10 s. Background images collected at the beginning of experiments at each excitation wavelength from adjacent cell-free fields were subtracted and acquired images divided on a pixel-by-pixel basis to provide ratioed images. $[Ca^{2+}]_i$ was estimated using external calcium/fura-2 standards as previously described (Ljungström and Chew, 1990). Since each experiment served as its own control, data in figures is expressed as 340/380 ratio values which have been shown to be highly reproducible from experiment to experiment utilizing experimental conditions described here and in an earlier publication (Ljungström and Chew, 1990).

Statistical analyses

Where applicable, data are expressed as means ± SEM, with n = the number of cellular preparations isolated from different animals. Statistical analyses of data were performed using analysis of variance and Dunnett's and Duncan's multiple range tests.

Acknowledgments

We thank Ms. Cynthia Allen for excellent technical assistance. This work was supported by National Institutes of Health Grants, DK31900, RR03825, and RR03034.

Received: August 1, 1990.
Revised and accepted: October 22, 1990.

References

Ali, H., Christensen, S.B., Foreman, J.C., Pearce, F.L., Piotrowski, W., and Thastrup, O. (1985). The ability of thapsigargin and thasigargicin to activate cells involved in the inflammatory response. Br. J. Pharmacol. *85,* 705–712.

Anderson, N.G., and Hanson, P.J. (1984). Inhibition of gastric acid secretion by a phorbol ester: effect of 12-O-tetradecanoyl-phorbol-13-acetate on aminopyrine accumulation by

rat parietal cells. Biochem. Biophys. Res. Commun. *121,* 566–572.

Berglindh, T., and Obrink, K.J. (1976). A method for preparing isolated glands from the rabbit gastric mucosa. Acta Physiol. Scand. *96,* 150–159.

Berglindh, T., Sachs, G., and Takeguchi, N. (1980). Ca^{2+}-dependent secretagogue stimulation in isolated rabbit gastric glands. Am. J. Physiol. *239*(Gastrointest. Liver Physiol. *2*), G90–94.

Brayden, D.J., Hanley, M.R., Thastrup, O., and Cuthbert, A.W. (1989). Thapsigargin, a new calcium-dependent epithelial anion secretagogue. Br. J. Pharmacol. *98,* 809–816.

Brown, M.R., and Chew, C.S. (1987). Multiple effects of phorbol ester on secretory activity in rabbit gastric glands and parietal cells. Can. J. Physiol. Pharmacol. *65,* 1840–1847.

Brown, M.R., and Chew, C.S. (1989). Carbachol-induced protein phosphorylation in parietal cells: regulation by $[Ca^{2+}]_i$. Am. J. Physiol. *257*(Gastrointest. Liver Physiol. *20*), G99–110.

Cheek, T.R., and Thastrup, O. (1989). Internal Ca^{2+} mobilization and secretion in bovine adrenal chromaffin cells. Cell Calcium *10,* 213–221.

Chew, C.S., Sachs, G., Hersey, S.J., and Berglindh, T. (1980). Histamine responsiveness of isolated gastric glands. Am. J. Physiol. *238*(Gastrointestin. Liver Physiol. *1*), G312–320.

Chew, C.S., and Hersey, S.J. (1982). Gastrin stimulation of isolated gastric glands. Am. J. Physiol. *242*(Gastrointest. Liver Physiol. *5*), G504–512.

Chew, C.S. (1985a). Parietal cell protein kinases. Selective activation of type I cAMP-dependent protein kinase by histamine. J. Biol. Chem. *260,* 7540–7550.

Chew, C.S. (1985b). Differential effects of extracellular calcium removal and nonspecific effects of Ca^{2+} antagonists on acid secretory activity in isolated gastric glands. Biochim. Biophys. Acta *846,* 370–378.

Chew, C.S., and Brown, M.R. (1986). Release of intracellular Ca^{2+} and elevation of inositol triphosphate by secretagogues in parietal and chief cells isolated from rabbit gastric glands. Biochim. Biophys. Acta *888,* 116–125.

Chew, C.S. (1989). Intracellular activation events for parietal cell HCl secretion. In: Handbook of Physiology, Section 6: The Gastrointestinal System, ed. J.G. Forte, New York: Oxford University Press, 255–266.

Chew, C.S., Ljungström, M., Smolka, A., and Brown, M.R. (1989). Primary culture of secretagogue-responsive parietal cells from rabbit gastric mucosa. Am. J. Physiol. *256*(Gastrointest. Liver Physiol. *19*), G254–263.

Chew, C.S., and Ljungström, M. (1990). HCl secretion and $[Ca^{2+}]_i$ in cultured parietal cells. J. Intern. Med. *228*(suppl. 732), 9–15.

Christensen, S.B., Larsen, I.K., and Rasmussen, U. (1982). Thapsigargin and thapsigarcin, two histamine liberating sequiterpene lactones from *Thapsia gargancia.* X-ray analysis of the 7,11-epoxide of thapsigargin. J. Org. Chem. *47,* 649–652.

Demarest, J.R., and Loo, D.F. (1990). Electrophysiology of the parietal cell. Annu. Rev. Physiol. *52,* 307–319.

DiBona, D.R., Ito, S., Berglindh, T., and Sachs, G. (1979). Cellular site of gastric acid secretion. Proc. Natl. Acad. Sci. USA *76,* 6689–6693.

Foder, B., Scharff, O., and Thastrup, O. (1989). Ca^{2+} transport and Mn^{2+} entry in human neutrophils induced by thapsigargin. Cell Calcium *10,* 477–490.

Forte, J.G., and Soll, A.H. (1989). Cell biology of hydrochloric acid secretion. In: Handbook of Physiology, Section 6: The Gastrointestinal System, ed. J.G. Forte, New York: Oxford University Press, 207–228.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260,* 3440–3450.

Hakii, H., Fujiki, H., Suganuma, M., Nakayasu, M., Tahira, T., Christensen, S.B., Scheuer, P.J., and Sugimura, T. (1986). Thapsigargin, a histamine secretagogue, is a non-12-O-tetradecanoyl-phorbol-13-acetate (TPA) type tumor promoter in two-stage mouse carcinogenesis. J. Cancer Res. Clin. Oncol. *111,* 177–181.

Jackson, T.P., Patterson, S.I., Thastrup, O., Hanley, M.R. (1988). A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells. Biochem. J. *253,* 81–86.

Jiron, C., Ruiz, M.C., and Michelangeli, F. (1981). Role of Ca^{2+} in stimulus-secretion coupling in the gastric oxyntic cell: effect of A23187. Cell Calcium *2,* 573–585.

Kano, S., Iizuka, T., Ishimura, Y., Fujiki, H., and Sugimura, T. (1987). Stimulation of superoxide anion formation by the non-TPA type tumor promoters palytoxin and thapsigargin in porcine and human neutrophils. Biochem. Biophys. Res. Commun. *143,* 672–677.

Ljungström, M., and Chew, C.S. (1990). Calcium oscillations and morphological transformations in single cultured gastric parietal cells. Am. J. Physiol. (in press).

Muallem, S., and Sachs, G. (1985). Ca^{2+} metabolism during cholinergic stimulation of acid secretion. Am. J. Physiol. *248*(Gastrointest. Liver Physiol. *11*), G216–228.

Muallem, S., Fimmel, C.J., Pandol, S.J., and Sachs, G. (1986). Regulation of free cytosolic Ca^{2+} in the peptic and parietal cells of the rabbit gland. J. Biol. Chem. *261,* 2660–2667.

Michelangeli, F., Ruiz, M.C., Fernandez, E., and Ciarrocchi, A. (1989). Role of Ca^{2+} in H^+ transport by rabbit gastric glands studied with A23187 and BAPTA, an incorporated Ca^{2+} chelator. Biochim. Biophys. Acta *983,* 82–90.

Negulescu, P.A., and Machen, T.E. (1988). Release and reloading of intracellular Ca stores after cholinergic stimulation of the parietal cell. Am. J. Physiol. *254*(Cell Physiol *23*), C498–504.

Negulescu, P.A., Reenstra, W.W., and Machen, T.E. (1989). Intracellular Ca requirements for stimulus-secretion coupling in parietal cell. Am. J. Physiol. *256*(Cell Physiol. *25*), C241–251.

Noar, Z. (1990). Further characterization of protein kinase-C subspecies in the hypothalamo-pituitary axis: differential activation by phorbol esters. Endocrinology *126,* 1521–1526.

Nylander O., Bergqvist, E., and Obrink, K.J. (1985). Dual inhibitory actions of somatostatin on isolated gastric glands. Acta Physiol. Scand. *125,* 111–119.

Ohuchi, K., Sugawara, T., Watanabe, M., Hirasawa, N., Tsurufuji, S., Fujiki, H., Christensen, S.B., and Sugimura, T. (1988). Analysis of the stimulative effect of thapsigargin, a non-TPA-type tumor promotoer, on arachidonic acid metabolism in rat pertoneal macrophages. Br. J. Pharmacol. *94,* 917–923.

Ohuchi, K., Takahashi, C., Hirasawa, N., Watanabe, M., Fujiki, H., Tsurufuji, S. (1989). Stimulation of histamine release and arachidonic acid metabolism in rat peritoneal mast cells by thapsigargin, a non-TPA-type tumor promoter. Biochim. Biophys. Acta *1003,* 9–14.

O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge, M.J., and Burgoyne, R.D. (1989). Localization and heterogeneity of agonist-induced changes in cytosolic calcium concentration in single bovine adrenal chromaffin cells from video-imaging of fura-2. EMBO J. *8,* 401–411.

Park, Y., Chiba, T., and Yamada, T. (1987). Mechanism for direct inhibition of canine gastric parietal cells by somatostatin. J. Biol. Chem. *262,* 14190–14196.

Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S., Webster, C. (1989). Protein kinase C—a family affair. Mol. Cell. Endocrinol. *65,* 1–11.

Putney, J.W. (1986). A model for receptor-regulated calcium entry. Cell Calcium *7,* 1–12.

Scharff, O., Foder, B., Thastrup, O., Hofmann, B., Møller, J., Ryder, L.P., Jacobsen, K.D., Langhoff, E., Dickmeiss, E., Christensen, S.B., Skinnhøj, P., and Svejgaard, A. (1988). Effect of thapsigargin on cytoplasmic Ca^{2+} and proliferation of human lymphocytes in relation to AIDS. Biochim. Biophys. Acta *972,* 257–264.

Soll, A.H. (1981). Extracellular calcium and cholinergic stimulation of isolated canine parietal cells. J. Clin. Invest. *68,* 270–278.

Soll, A.H., and Berglindh, T. (1987). Physiology of isolated gastric glandsand parietal cells: receptors and effectors regulating function. In: Physiology of the Gastrointestinal Tract, 2nd ed., vol. 1, ed. L.R. Johnson, New York: Raven Press, 883–909.

Takemura, H., Thastrup, O., and Putney, J.W. Jr. (1990). Calcium efflux across the plasma membrane of rat parotid acinar cells is unaffected by receptor activation or by the microsomal calcium ATPase inhibitor, thapsigargin. Cell Calcium *11,* 11–17.

Thastrup, O., Foder, B., and Scharff, O. (1987). The calcium mobilizing and tumor promoting agent, thapsigargin elevates the platelet cytoplasmic free calcium concentration to a higher steady state level. A possible mechanism of action for the tumor promotion. Biochem. Biophys. Res. Commun. *142,* 654–660.

Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R., and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA *87,* 2466–2470.