

Calcium Signaling Mechanisms in the Gastric Parietal Cell

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Gastric hydrochloric acid (HCl) secretion is stimulated *in vivo* by histamine, acetylcholine, and gastrin. *In vitro* studies have shown that histamine acts mainly via a cAMP-dependent pathway, and acetylcholine acts via a calcium-dependent pathway. Histamine also elevates intracellular calcium ($[Ca^{2+}]_i$) in parietal cells. Both gastrin and acetylcholine release histamine from histamine-containing cells. In humans, rats, and rabbits, there is considerable controversy as to whether or not gastrin receptors are also present on the parietal cell. We utilized digitized video image analysis techniques in this study to demonstrate gastrin-induced changes in intracellular calcium in single parietal cells from rabbit in primary culture. Gastrin also stimulated a small increase in $[^{14}C]$ -aminopyrine (AP) accumulation, an index of acid secretory responsiveness in cultured parietal cells. In contrast to histamine and the cholinergic agonist, carbachol, stimulation of parietal cells with gastrin led to rapid loss of the calcium signaling response, an event that is presumed to be closely related to gastrin receptor activation. Moreover, different calcium signaling patterns were observed for histamine, carbachol, and gastrin. Previous observations coupled with present studies using manganese, caffeine, and ryanodine suggest that agonist-stimulated increases in calcium influx into parietal cells do not occur via voltage-sensitive calcium channels or nonspecific divalent cation channels. It also appears to be unlikely that release of intracellular calcium is mediated by a muscle or neuronal-type ryanodine receptor. We hypothesize that calcium influx may be mediated by either a calcium exchange mechanism or by an unidentified calcium channel subtype that possesses different molecular characteristics as compared to muscle, nerve, and certain secretory cell types such as, for example, the adrenal chromaffin cell. Release of intracellular calcium may be mediated via both $InsP_3$ -sensitive and -insensitive mechanisms. The $InsP_3$ -insensitive calcium pools, if present, do not appear, however, to possess ryanodine receptors capable of modulating calcium efflux from these storage sites.

INTRODUCTION

Because peptic ulcer disease is prevalent world-wide and gastric acid secretion has been implicated in the progression of this disease, the source of hydrochloric acid (HCl) secretion, the gastric parietal cell, has been intensively studied. *In vivo*, gastric acid secretion is stimulated by neural, paracrine, and endocrine events that involve three classes of agonists, histamine, acetylcholine, and gastrin, all of which when administered separately are potent stimulators of gastric HCl secretion. Histamine H_2 and muscarinic m_3 -type receptors have been localized to parietal cells in several species, including human. Because gastrin has either a small or no stimulatory effect

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Abbreviations: AF: autofluorescence AP: aminopyrine BSA: bovine serum albumin $[Ca^{2+}]_i$: intracellular calcium carbachol: carbamylcholine chloride CCK-8: cholecystokinin octapeptide CICR: calcium-induced calcium release DME: Dulbecco's modified Eagle's medium ECL: enterochromaffin-like (cells) HCl: hydrochloric acid $InsP_3$: inositol 1,4,5-trisphosphate ISIT: intensified silicon intensifier target Mn: manganese PBS: phosphate-buffered saline

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on acid secretory-related activities in isolated parietal cells, however, there has been considerable controversy as to whether or not gastrin receptors are present on parietal cells of rats, rabbits, and humans [1–3]. In contrast, it is generally accepted that these receptors are present on canine parietal cells, and a gastrin receptor with characteristics similar to the CCK-B-type receptor has recently been cloned from a canine cDNA library [4]. Like gastrin, the efficacies of histamine and cholinergic analogs such as carbachol vary among different species both *in vivo* and *in vitro*. In humans and rabbits, for example, histamine is more efficacious than carbachol, whereas the reverse is true for the dog [1–3,5].

The parietal cell responds to secretory agonists with complex morphological transformations that involve fusion of intracellular vesicles containing the acid secreting proton “pump” or H,K ATPase with the apical canalicular membrane. A currently held belief is that the insertion of the H,K ATPase into the canalicular membrane is the final intracellular event leading to increased HCl secretion [5]. The intracellular signaling events that occur prior to insertion of the “pump” into the apical membrane involve both calcium and cAMP-dependent mechanisms. It is established that histamine activates the enzyme, adenylyl cyclase, via an H₂-receptor interaction with resultant elevation of cellular cAMP content and activation of cAMP-dependent protein kinase(s). Gastrin, cholecystokinin octapeptide (CCK-8), and the acetylcholine analog, carbachol, have been reported to elevate [Ca²⁺]_i in parietal cells from some species, but do not detectably alter cellular cAMP content [1–3,5]. Interestingly, histamine also elevates intracellular free calcium concentrations ([Ca²⁺]_i) in parietal cells by an H₂-receptor-dependent but cAMP-independent mechanism [6–10]. Moreover, when cloned histamine H₂ receptor cDNA is expressed in COS cells, histamine addition elevates both cAMP content and [Ca²⁺]_i [11].

The specific cellular activation events that occur following elevation of [Ca²⁺]_i and cAMP are not well defined. Several studies have detected increased phosphorylation of specific intracellular proteins in response to histamine and agents that elevate intracellular cAMP content, such as dbcAMP and forskolin. Carbachol and CCK-8/gastrin increase phosphorylation of a different set of proteins [12–15]. Since histamine also elevates [Ca²⁺]_i, it is somewhat surprising that phosphoprotein studies have not detected any overlap in phosphorylation patterns. Functional experiments with isolated parietal cells and gastric glands have also suggested, however, that calcium plays either a minor or no role in the direct modulation of histamine-stimulated HCl secretion. In contrast, calcium appears to play a major role in the mediation of potentiating interactions between histamine and calcium-dependent agonists [1,3,16,17].

This study was initiated to determine whether differences in calcium signaling patterns and/or different intracellular sources of calcium in combination with varying calcium influx pathways might explain the variations in secretory responses to histamine, carbachol, and gastrin. A video camera-based digitized image analysis system was used to define calcium signaling mechanisms in single parietal cells in primary culture [16–18]. Acutely isolated, highly enriched parietal cells were also used for fluorimeter-based cell population experiments aimed at determining whether calcium influx mechanisms are similar to or different from those observed in other secretory cell types. Data from this study in conjunction with previously published observations suggest that calcium does not enter parietal cells by either nonspecific

divalent cation channels or classic voltage-sensitive channels that are blocked by L-type calcium channel antagonists. Release of intracellular calcium may occur by inositol 1,4,5-trisphosphate (InsP₃)-sensitive and -insensitive mechanisms but, under standard experimental conditions, is unaffected by either ryanodine or caffeine, which are agents that modulate intracellular calcium release in muscle, neurons, and certain secretory cell types [19–25]. Measurements of changes in $[Ca^{2+}]_i$ in single parietal cells from rabbits support the hypothesis that gastrin receptors are present but may be heterogeneously distributed. Moreover, calcium signaling patterns exhibit clear differences in response to histamine, carbachol, and gastrin. In response to gastrin, but not to histamine or carbachol, there is a rapid downregulation of the calcium response, an event that may partially explain a weak direct stimulatory effect of gastrin on parietal cells *in vitro*.

MATERIALS AND METHODS

Gastric mucosa from 1.5–3 Kg male New Zealand white rabbits was used as a starting material in all experiments. Ham's F12 and Dulbecco's modified Eagle's medium (DME) with I-glutamine and 15 mM HEPES, without bicarbonate, fetal calf serum, insulin-transferrin-sodium selenite, hydrocortisone, epidermal growth factor, gastrin (synthetic, Human I), histamine dihydrochloride, carbamylcholine chloride (carbachol), caffeine, amphotericin-B, gentamicin, geneticin, novobiocin sulfate, cimetidine, bovine serum albumin, fraction V (BSA, endotoxin-free), and collagenase, Type II, were purchased from Sigma Chemical Company. Fura-2, fura-2/AM, ryanodine (high purity), and ionomycin were from Calbiochem. Nycodenz (sterile isotonic solution) was purchased from Accurate Chemical; Matrigel[®] and Pronase (protease, *Streptomyces griseus*, specific activity, 103 U/mg) from Collaborative Research; [¹⁴C]-aminopyrine (AP; 10 mCi/mmol) from New England Nuclear. Research-grade water (Modulab Water System, Continental Water Co.) was used in all procedures.

Parietal Cell Isolation, Enrichment, and Primary Culture

Parietal cells were isolated, enriched to > 90 percent purity, and placed in primary culture as previously described [18]. In brief, the gastric mucosa of male New Zealand white rabbits (1.5–3 kg) was perfused *in situ* with phosphate-buffered saline (PBS) under high pressure, then sequentially digested with pronase followed by collagenase to produce single cells. Nycodenz gradient separation, followed by centrifugal elutriation with a Beckman JE 5.0 elutriator rotor, was used for parietal cell enrichment. Following elutriation, cells were washed twice by brief centrifugation and resuspension in medium A (1:1 mixture of Ham's F12 and DME (pH 7.4), BSA (2 mg/ml); epidermal growth factor (8 nM), hydrocortisone (10 nM), insulin (800 nM), transferrin (5 µg/ml), sodium selenite (5 ng/ml), glucose (10 mM), novobiocin (50 µg/ml), geneticin (5 µg/ml), and gentamicin sulfate (100 µg/ml). Cells were then pre-attached in medium A containing amphotericin-B (25 µg/ml, 30 minutes, 37°C in a 75 cm² Corning polystyrene tissue culture flask that had been pre-coated for 24 hours with 10 percent calf serum) to remove contaminating fibroblasts and to reduce yeast contamination. Cells were routinely plated on a thin layer of dried Matrigel (50 µl, 1:7 dilution) in medium A without amphotericin-B at a density of ~10⁵ cells/dish in a total volume of 1.5 ml and incubated in a humidified air incubator.

Estimation of HCl Secretion with [¹⁴C]-Aminopyrine

Acid secretory activity in cultured parietal cell populations was measured, using a modification [18] of the Berglindeh technique [26], which is based on the observation that aminopyrine, a weak base with a pKa of 5, accumulates within acidic vesicular compartments in parietal cells due to protonation at pHs below 5. Thirty minutes prior to agonist addition, attached cells were rinsed and pre-incubated in medium B (in mmol/l: 114.4 NaCl, 5.4 KCl, 5.0 Na₂HPO₄, 1.0 NaH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, 10.0 glucose, 1.0 pyruvate, 20.0 HEPES, pH 7.4; BSA (2 mg/ml) and phenol red (10 mg/ml) and then incubated an additional 30 minutes in medium B containing [¹⁴C]-aminopyrine (AP, 0.1 μCi/ml). Cells were exposed to agonists for indicated amounts of time. When agonists other than histamine were used, the H₂-receptor antagonist, cimetidine (10 μM), was included in the incubation to block the effect of any histamine that might be present in the culture. Thiocyanate (10 mM) was added to separate dishes in order to correct for nonspecific trapping of AP. Incubations were terminated by rapidly removing the supernatant under vacuum, rinsing the cells twice with PBS, and lysing cells with 1 ml of PBS containing 1 percent Triton X-100. Aliquots of cell lysates and supernatants were counted in Ecolite (Westchem) in a Beckman LS6800 liquid scintillation counter with DPM correction. AP accumulation ratios were calculated, based on cell number as previously described [18].

Digitized Video Image Analysis of [Ca²⁺]_i in Single Cells and Fluorimetric Measurements in Cell Populations

The calcium-sensitive fluorescent probe, fura-2, was used in all experiments to define relative changes in [Ca²⁺]_i. For cell population measurements, a temperature-controlled (37°C) Perkin-Elmer 650-40 spectrofluorimeter was employed, as previously described [6]. To facilitate fluorescent/transmitted light digitized video image acquisitions, cells were cultured on 25 mm glass coverslips that were thinly coated with Matrigel [16]. Changes in [Ca²⁺]_i were measured, using the dual wavelength excitation ratio technique [16,27,28]. Cells were loaded with fura-2 by incubating in medium B containing the cell-permeant form (fura-2/AM, 4 μM, 30 minutes) in a humidified 37°C air incubator, rinsed twice with medium B, and placed in a temperature-controlled (37°C) stage of a modified Zeiss IM35 microscope mounted on a vibration-free air table (Kinetic Systems, Boston, MA). Fura-2 fluorescence in single cells was quantitated with an ISIT (intensified silicon intensifier target, DAGE 66) video camera (manual gain and voltage control, defeated gamma correction) mounted on the side port of the microscope or with a DAGE-MTI Genllsys camera (VE1000) on the top port. Excitation wavelengths were selected with a computer-controlled filter wheel (LEP, Hawthorne, NY). Ratioed images of fura-2 fluorescence were acquired with a Nikon 100x (UV-F, 1.3 N.A.) glycerol immersion objective at 340/380 nm excitation and 510 nm emission wavelengths, using narrow bandpass excitation filters (Omega Optical, 340 and 380 DF 10) in conjunction with 405 nm dichroic beam splitter and 510 nm LP emission filter, quartz nosepiece and 3 lens collector, and quartz heat protection and neutral density filters to attenuate 85–97 percent of the excitation light. Background fluorescence images for both excitation wavelengths were acquired at the beginning of each experiment from a blank field in the same focal plane as the cells and subtracted pixel by pixel from each wavelength pair prior to ratioing. Camera and computer-controlled gains were

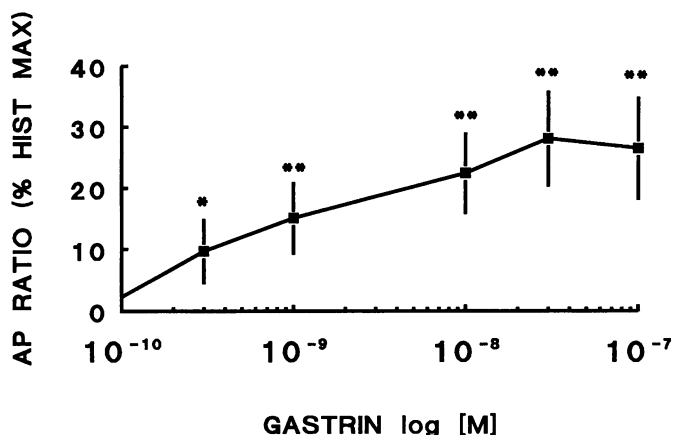


FIG. 1. Gastrin increases AP accumulation in parietal cells in primary culture in a dose-related manner. Parietal cells isolated from rabbit gastric mucosa were cultured on a thin coating of Matrigel for three days and then stimulated with several different concentrations of gastrin for 30 minutes. AP accumulation, which is an indirect measure of acid secretory activity, was then measured, as described in Methods, and expressed as a percentage of the response to 10 μ M histamine by cells in the same preparations. Basal AP accumulation was $2,204 \pm 285$. Histamine-stimulated AP accumulation was $4,805 \pm 505$. Values are means \pm SEMs for $n = 4-5$ preparations (* $p < 0.05$; ** $p < 0.01$ statistically different from basal). The apparent EC_{50} for gastrin-stimulated AP accumulation was $6.5 \pm 1.9 \times 10^{-10}$ M.

adjusted so that autofluorescence (AF) from cells prior to fura loading was undetectable. Changes in $[Ca^{2+}]_i$ were expressed as 340/380 nm ratios rather than as absolute calcium concentrations, due to uncertainties surrounding the calibration of intracellular free calcium concentrations with the fura-2 technique [16]. Images were acquired at eight-bit resolution and analyzed at 16-bit resolution with shading correction using Invision IC300 software and a SUN 3/280S or a Sparc2 minicomputer. In routine experiments, 16–32 frames were averaged for each excitation wavelength with a 12-frame (375 msec) delay between samples to avoid sampling bias.

RESULTS

Gastrin Increases AP Accumulation in Cultured Parietal Cells

Figure 1 shows that gastrin stimulates a small increase in AP accumulation in parietal cells maintained in primary culture. The apparent EC_{50} for four separate preparations assayed in the presence of 10 μ M cimetidine to block the action of any endogenous histamine that might be present was $6.5 \pm 1.9 \times 10^{-10}$ M. These results confirm and extend previous observations that gastrin stimulates AP accumulation in cultured as well as acutely isolated parietal cells and gastric glands from rabbits [18,29]. Although a response to gastrin is more readily demonstrated in cultured cells than in acutely isolated cells, possibly because receptors have a chance to recover from damage induced by digestive enzymes required for cell isolation, the magnitude of the AP response in both models was considerably less than that to histamine or

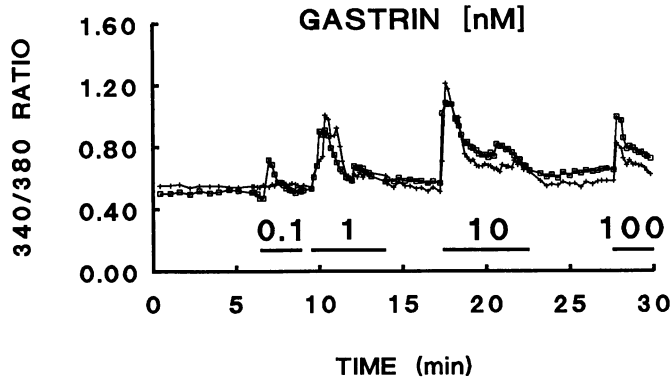


FIG. 2. Gastrin stimulation leads to heterogeneous increases in $[Ca^{2+}]_i$ and tachyphylaxis in cultured parietal cells. Cells were isolated, enriched, cultured on Matrigel, then loaded with fura-2, and subjected to single-cell image analysis, as described in Methods. In this experiment, four cells were imaged simultaneously. Only one of the four responded to 0.1 nM gastrin, whereas all responded to 1 nM. The calcium response to 10 nM gastrin was greater than that to 1 nM, but subsequent stimulation with 100 nM gastrin led to an attenuated response. Horizontal bars in the figure indicate times of agonist addition and length of exposure. In other experiments, ~30 percent of cells ($n = 57$, six animals) did not respond to a gastrin challenge but did respond to a subsequent carbachol challenge. Calcium oscillations in response to gastrin were observed in 27 percent of responding cells.

carbachol. In this study, the maximal gastrin response was ~25–30 percent of the maximal histamine response, whereas the carbachol response was ~60 percent of the maximal histamine response ($n = 4-5$). In acutely isolated parietal cells and glands, previous results indicated the gastrin response to be ~10–15 percent of the histamine response with an approximate EC_{50} of 5 nM [29; unpublished observations].

Gastrin Stimulates Heterogeneous Increases in $[Ca^{2+}]_i$

In experiments in which 57 cells from five different animals were tested for effects of gastrin on intracellular free calcium concentrations, a maximal stimulatory dose of gastrin (10–100 nM) was found to elevate $[Ca^{2+}]_i$ in 40 of these cells, whereas 100 percent of the cells responded to a subsequent carbachol challenge. Twenty-seven percent of the cells that responded to gastrin exhibited oscillations in the calcium response. When submaximal doses of gastrin were administered, fewer cells responded (six and 24 of the 57 cells responded, respectively, to 0.1 and 1 nM gastrin). Moreover, stimulation with increasing concentrations of gastrin led to a diminished calcium response. Figure 2 depicts responses of two such cells that were imaged simultaneously. Note that only one cell responded to 0.1 nM gastrin and, although both responded to 1–100 nM gastrin, the response to 100 nM gastrin was greatly attenuated, as compared to the response at 10 nM. In other experiments (not shown), 100 nM gastrin alone elevated $[Ca^{2+}]_i$ to levels similar to those observed with 10 nM gastrin. Thus, the reduced response to 100 nM gastrin shown in Fig. 2 was not simply due to a diminished calcium response. The onset of tachyphylaxis was somewhat variable from cell to cell. In Fig. 3, for example, administration of a single

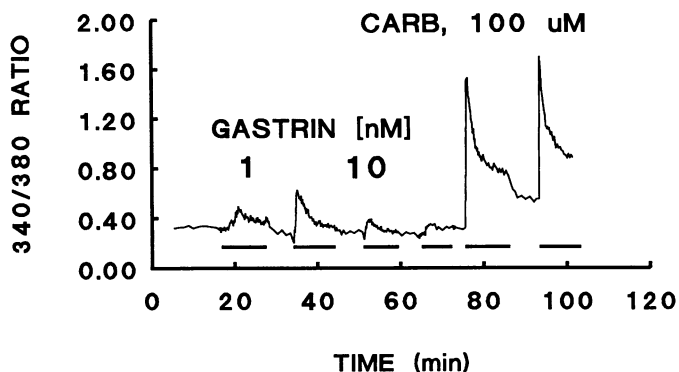


FIG. 3. Maximal to near-maximal doses of gastrin can rapidly suppress the calcium response to subsequent gastrin challenges. In contrast, no short-term tachyphylaxis is detectable with carbachol. Parietal cells were isolated, placed in primary culture, and treated as described in Fig. 2. Cells were stimulated with a submaximal dose of gastrin (1 nM), then repetitively stimulated (3 \times) with a near maximal dose (10 nM). Following the initial addition and removal of 10 nM gastrin, the calcium response was attenuated. In other experiments (not shown), the response was completely abolished following addition of 100 nM gastrin. The degree of tachyphylaxis varied from cell to cell but was always observed in multiple experiments conducted over a period of several months.

10 nM dose of gastrin completely abolished further responses to this same dose. In contrast, the response of the same cell to repetitive maximal doses of carbachol (100 nM) was unchanged. Since the number of cells responding to low doses of gastrin varied from cell to cell and since there was substantial tachyphylaxis, particularly at higher doses of gastrin, it was not possible to obtain an EC_{50} for gastrin-stimulated increases in $[Ca^{2+}]_i$ in single parietal cells.

Calcium Response Patterns Are Different for Gastrin, Histamine, and Carbachol

Comparisons of the patterns of change in $[Ca^{2+}]_i$ in single parietal cells indicated that the rise in calcium in response to gastrin declined more rapidly to basal or near basal levels as compared to carbachol or histamine. Figure 4 shows changes in $[Ca^{2+}]_i$ in a parietal cell stimulated with 0.1 nM and then 10 nM gastrin, followed by 100 μ M histamine, then 100 μ M carbachol. The calcium transient in response to 0.1 nM gastrin was barely detectable. Ten nanomolar gastrin elicited a large spike in $[Ca^{2+}]_i$ that rapidly returned to near basal levels. In contrast, the initial spike elicited by carbachol was followed by a characteristic sustained plateau that was well above basal. The cell responded to histamine with repetitive oscillations that were smaller in magnitude than the initial spike. In a previous publication, we observed that ~ 30 percent of cells responding to histamine exhibited oscillatory behavior, whereas the majority responded with a single spike followed by a lower, sustained plateau [16].

Agonist-Dependent Calcium Entry and Release Mechanisms

Although there is substantial evidence that both release of calcium from intracellular stores and influx of calcium across the plasma membrane are involved in agonist-mediated increases in $[Ca^{2+}]_i$, the mechanisms involved in these changes are poorly understood. In an attempt to learn more about the mechanisms involved in

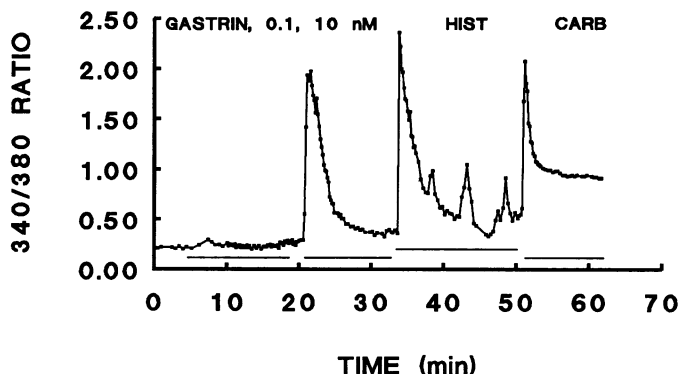


FIG. 4. Comparison of calcium response patterns elicited by gastrin, histamine, and carbachol in a single cultured parietal cell. Experimental conditions were similar to those described in Fig. 2. Gastrin concentrations are indicated in the figure. Histamine and carbachol were administered at a concentration of 100 μ M. Calcium signaling patterns in this figure are typical for maximal doses of gastrin and carbachol. The oscillations observed with histamine were detected in \sim 30 percent of cells in sampled populations [16]. In \sim 70 percent of cells, there was a simple biphasic rise in $[Ca^{2+}]_i$. In these cells, the plateau in $[Ca^{2+}]_i$ was typically higher than that for gastrin.

the differing calcium responses to gastrin, histamine, and carbachol, several agents that affect calcium release or influx in certain other cell types were tested. In the first set of experiments, we sought to determine whether or not the agonist-stimulated calcium entry pathways were similar to or different from those detected in, for example, platelets and possibly in pancreatic acinar cells [30,31], i.e., cell types in which experiments with the divalent cation, manganese (Mn), have suggested that calcium-dependent agonists increase calcium entry via divalent cation "channels." Since Mn quenches fura-2 fluorescence, increased Mn influx into fura-2-loaded cells may be measured as a reduction in fura-2 fluorescence. In such experiments, Mn entry is detected using a single wavelength because the ratiometric technique will cancel out quenching, as it does photobleaching [27,28].

When single parietal cells were monitored in the presence of extracellular Mn, no significant Mn entry (i.e., quenching of fura-2 fluorescence) was detected either in stimulated or unstimulated cells monitored at both 340 and 380 nm wavelengths (Fig. 5). A similar protocol was performed with populations of fura-2-loaded cells in a spectrofluorimeter, except that, in these experiments, fluorescence at 340 nm and 360 nm (the isosbestic point for fura-2) was monitored. At the isosbestic point, the fura-2 signal does not change in response to changes in calcium concentrations as it does at 340 nm (increases) and at 380 nm (decreases). Thus, a decrease in fluorescence at 360 nm reflects either dye photobleaching or quench. In cell populations, but not perfused single cells, fura-2 fluorescence may be quenched as a result of leakage from cells or influx of extracellular Mn into cells. Figure 6 depicts several recordings made from the same population of fura-2-loaded parietal cells in the presence and absence of extracellular Mn at 340 and 360 nm wavelengths. In the top recording, responses to 100 μ M histamine followed by 100 μ M carbachol at 340 nm are shown. In the second recording, the effects of Mn on responses to these

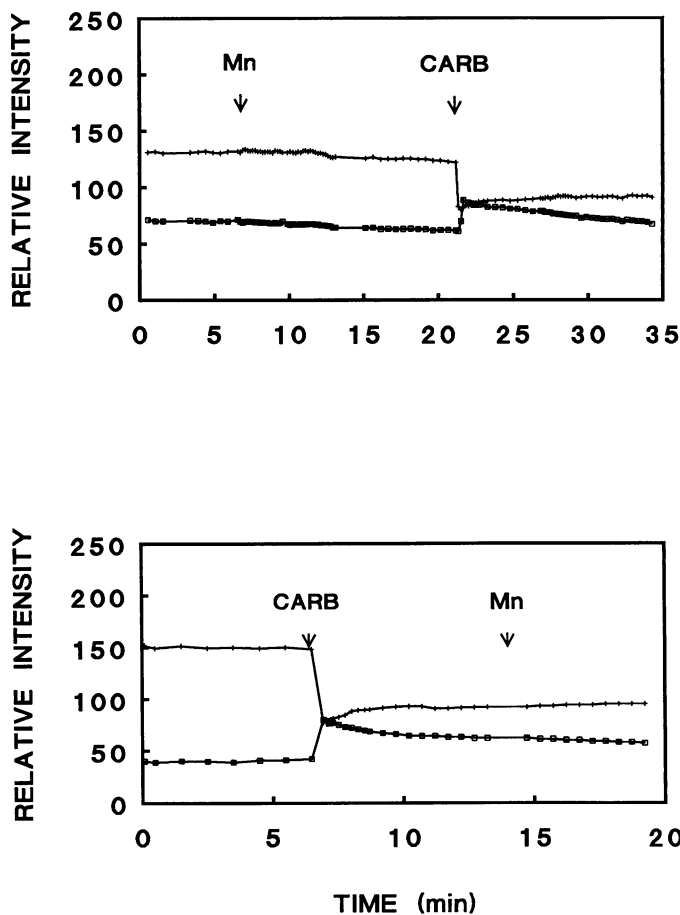


FIG. 5. In single parietal cells, Mn has no effect on fura-2 fluorescence intensity under basal or stimulated conditions. Parietal cells in primary culture were loaded with fura-2 and subjected to video image analysis, as described in Methods. Fluorescence intensity at 340 nm (lower recording) and 380 nm (upper recording) excitation λ 's wavelengths is expressed on a grey level scale of 0–255. Intensity values were used to demonstrate that when cells are perfused, Mn (0.5 mM) addition does not alter fluorescent intensity or the pattern of change in intensity observed following agonist addition (10 μ M carbachol in this experiment). Similar results were obtained with six different cellular preparations, in which 53 cells were imaged.

same agonists is depicted. Note that Mn addition results in an initial rapid quenching of the fluorescent signal, followed by a steady decline. Both events are most likely due to Mn quenching of extracellular fura-2, because no decrease in fura-2 fluorescence was detected when cells were perfused with media which rapidly remove fura-2 that leaks from cells (Fig. 5). The stability of the 340 and 380 nm signals in cultured cells suggests that (1) photobleaching is insignificant under the experimental conditions employed, and (2) in contrast to cells in suspension, there is minimal leakage of fura-2 from cells to medium.

With cells in suspension, addition of histamine followed by carbachol led to an initial increase in the rate of decline in fluorescence followed by a return to the same steady-state rate of decline. The initial agonist-induced increase in fluorescence quench is likely due to the superimposition of the secondary decline in $[Ca^{2+}]_i$ that occurs in the absence of Mn (top recording) on the steady rate of Mn quenching of the fura-2 signal. This hypothesis is supported by the experiment depicted in the third recording, in which the rate of quench recorded at 360 nm was unchanged after addition of histamine followed by carbachol. At this calcium-insensitive wavelength, only a change in Mn quenching of fura-2 resulting from increased Mn entry or fura-2 efflux should be detected. Thapsigargin, which releases calcium from intracellular

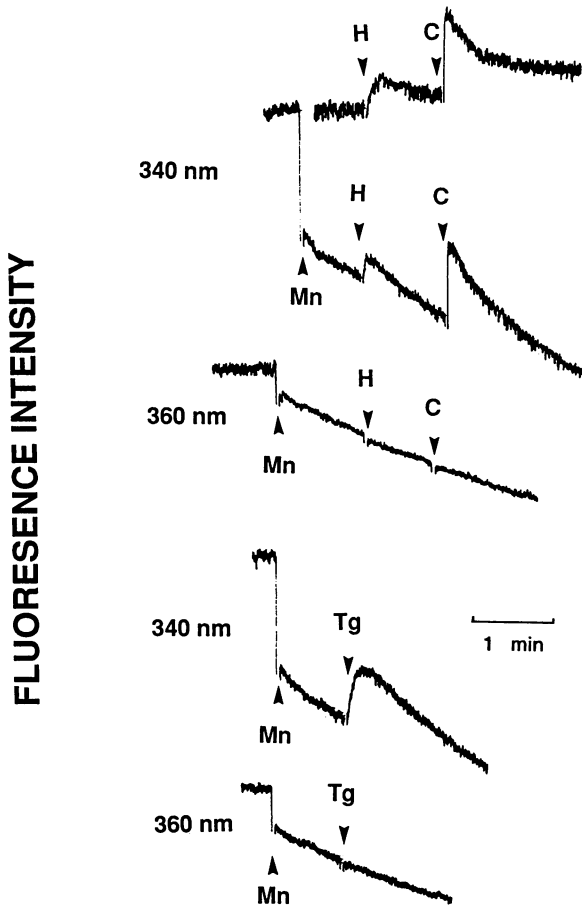


FIG. 6. Agonist-stimulated calcium entry into parietal cells does not appear to occur via a non-selective cation channel pathway. Acutely isolated parietal cells were loaded with 2 μ M fura-2 and fluorescence measured in a cuvette, as described in Methods. Arrows indicate additions. H, 100 μ M histamine; C, 100 μ M carbachol; Mn, 0.2 mM; T, thapsigargin, 1 μ M. Each recording represents a different sample of cells from the same population. The top recording was made at 340 nm excitation λ in the absence of Mn. Excitation λ s for recordings with Mn are to the left of each subsequent recording. The rapid quench in fura-2 fluorescence upon Mn addition is due to quenching of fura-2 in the extracellular medium. The steady decline in fluorescence is probably due to leakage of fura-2 from cells, because a similar decline is observed with the calcium chelator, EGTA (not shown). In this case, the fluorescent signal declines as fura-2 leaks from cells and enters a low-calcium environment, which leads to a reduction in the fura-2 signal. When the emission λ was set at 360 nm, the isobestic point for fura-2, agonist addition had no effect on the rate of decline in fura-2 fluorescence. Therefore, the initial rapid decline in fura-2 fluorescence upon agonist addition at 340 nm is the result of a superimposition of the decline observed in the absence of Mn (top recording) and Mn quenching of effluxed fura-2. These data and results from four additional preparations support the conclusion that agonists do not activate a nonspecific cation entry pathway that allows Mn entry.

stores by inhibition of the calcium re-uptake mechanism, a Ca-ATPase [32], also had no effect on the rate of Mn quench of fura-2 fluorescence recorded at either calcium-sensitive or -insensitive wavelengths (lowest two recordings). Similar results were obtained with gastrin (not shown). Thus, both single-cell data and cell population data suggest that agonist-stimulated calcium entry into parietal cells is unlikely to occur via non-selective cation channels.

In other experiments, caffeine and ryanodine were tested for possible effects on parietal cell calcium signaling events. In cultured parietal cells, neither caffeine (1–10 mM) nor ryanodine (1–10 μ M) had any detectable effect on basal $[Ca^{2+}]_i$ or on changes in $[Ca^{2+}]_i$ induced by histamine, carbachol, or gastrin when added up to 10 minutes before agonist addition or following agonist addition. Figure 7 depicts one

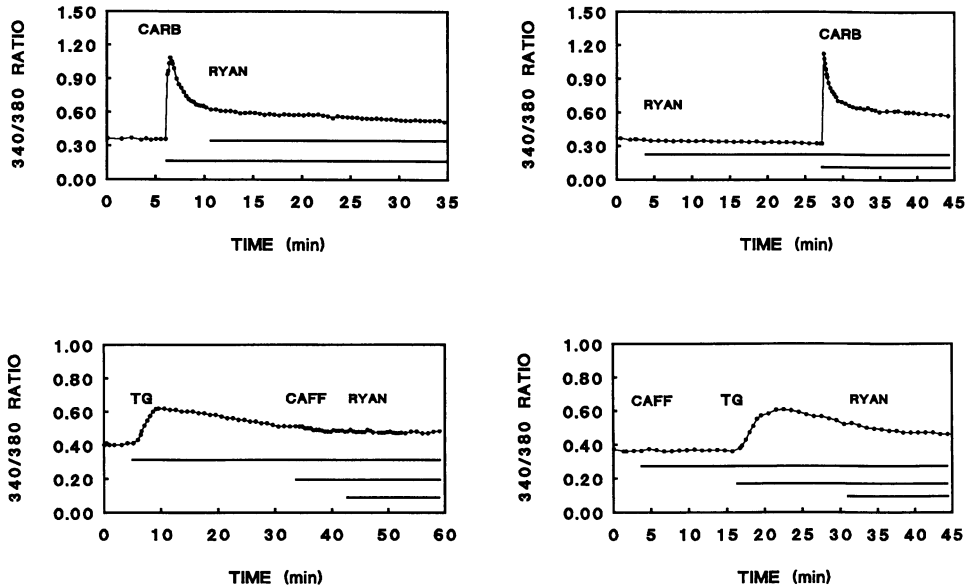


FIG. 7. Neither ryanodine nor caffeine affect basal or agonist-stimulated increases in parietal cell $[Ca^{2+}]_i$. Depicted here are four representative recordings obtained from single cultured parietal cells loaded with fura-2, as described in Methods and text. Similar results were obtained with four other cell preparations to which ryanodine was added at different times before or after carbachol or thapsigargin. Experimental conditions were similar to those in Fig. 2. CARB, 10 μ M carbachol; RYAN, 10 μ M ryanodine; TG, 2 μ M thapsigargin; CAFF, 10 mM caffeine.

such experiment, in which 10 μ M ryanodine was added either before or after 10 μ M carbachol (top two panels) or after addition of 2 μ M thapsigargin (lower two panels). Similar results were obtained with four other cell preparations (87 cells), to which ryanodine was added at different times before or after agonist stimulation. The two experiments in the lower panel indicate that caffeine also has no effect on calcium signaling patterns in parietal cells. These experiments were also repeated in four additional cell preparations with similar results. The experimental conditions depicted in the lower left panel of Fig. 7 were specifically chosen to illustrate the lack of effect of either caffeine or ryanodine on parietal cell calcium signaling patterns because they closely mimic experiments reported by Foskett and Wong [23], in which caffeine was reported to initiate oscillations in pancreatic acinar cell calcium signaling patterns when administered after thapsigargin, and ryanodine was reported to inhibit this effect.

DISCUSSION

This study is the first to demonstrate differences in calcium signaling patterns in response to gastrin, histamine, and carbachol within single cultured parietal cells. The data also indicate the presence, on the rabbit parietal cell, of a receptor that responds to gastrin by elevating $[Ca^{2+}]_i$ and AP accumulation. Since the AP accumulation experiments were conducted in the presence of the H_2 -receptor antagonist, cimetidine, and since the cells were >90 percent enriched, we conclude these gastrin effects are direct. Thus, the cultured cell data confirm previous observations on gastric glands and acutely isolated, enriched parietal cell popula-

tions in which it was found that gastrin and analogs elevate rabbit parietal cell $[Ca^{2+}]_i$, increase $InsP_3$ concentrations, and weakly increase AP accumulation [6,29,33]. A new finding is the rapid development of tachyphylaxis in gastrin-induced increases in parietal cell $[Ca^{2+}]_i$, a phenomenon that is particularly apparent at maximal to near maximal doses of gastrin. The tachyphylaxis observed with gastrin is in direct contrast to the apparent absence of short-term tachyphylaxis following administration of either the cholinergic agonists, as shown in this study, or of histamine [16]. The mechanism and significance of gastrin-induced tachyphylaxis is unclear. It may be that receptor downregulation is involved, and this downregulation might explain the weak direct action of gastrin on acid secretion. Another possibility is that gastrin is involved in the long-term regulation of parietal cell function, an event that is activated or terminated by receptor internalization. Thus, the heterogeneity of the calcium response to gastrin might be related to the state of parietal cell differentiation. *In vivo*, elevated plasma gastrin levels are associated with gastric mucosal hypertrophy in which there is an increase in parietal cell mass and an apparent increase in the number of histamine-containing or enterochromaffin-like (ECL) cells [34]. It is not yet established whether these changes are mediated by a direct effect of gastrin or indirectly by gastrin-induced release of other factor(s).

Some years ago, we proposed that gastrin exerts a dual action in rabbit by releasing histamine from paracrine cells and exerting a weak stimulatory effect directly on the parietal cell [29]. The cultured cell data support this hypothesis. We now further suggest that a similar mechanism exists in humans, but has been difficult to demonstrate due to cell damage in acutely isolated cells. In this study, we chose to utilize rabbit parietal cells in primary culture to investigate calcium signaling and acid secretory mechanisms because this model offers several advantages over acutely isolated cells, including improved agonist sensitivity and cell viability. The rabbit has an *in vivo* pattern of response to secretory agonists similar to humans [35]. Moreover, cultured parietal cells from rabbit retain the major phenotypic characteristics of cells *in vivo*, including an agonist-responsive proton "pump" or H,K-ATPase, the activation of which can be assessed by measurement of AP accumulation, a technique that is widely used to define acid secretory status in isolated glands and parietal cells [1-3,5]. The ability to monitor calcium signaling patterns in response to different agonists within the same cell is also an important advantage because such measurements cannot be made on cell populations suspended in a cuvette.

Although there is substantial evidence for an involvement of calcium in the activation of parietal cell HCl secretion, the intracellular mechanisms by which agonists elevate calcium and the characteristics of calcium signaling patterns in single cells have only recently begun to be investigated. With improved digitized video image analysis techniques, it has become apparent that calcium signaling patterns can vary from cell to cell and with different agonists. In the parietal cell, the differences in calcium signaling patterns initiated by gastrin as compared to carbachol and histamine may offer insights into the intracellular mechanisms involved in control of HCl secretion. Previous observations in single cultured parietal cells and parietal cell populations have suggested that histamine-stimulated increases in $[Ca^{2+}]_i$ are not correlated with acid secretory-related responses [1,3,16,17]. Thus, either histamine does not act on the same calcium pools as carbachol and gastrin or the calcium response pattern with histamine is inappropriate for regulation of secretion.

The generally accepted model for agonist-stimulated increases in intracellular calcium is that InsP_3 , which is produced in conjunction with diacylglycerol following agonist-stimulated, phospholipase C-catalyzed breakdown of phosphatidylinositol 4,5-bisphosphate, causes release of calcium from intracellular storage sites [36]. Since both carbachol and gastrin, but not histamine, induce a significant elevation in cellular inositol 1,4,5-trisphosphate (InsP_3) concentrations [6,33,37], this mechanism may not account for histamine-stimulated release of $[\text{Ca}^{2+}]_i$. The possibility that histamine-stimulated increases in InsP_3 do occur cannot be completely ruled out, however, because Gantz and colleagues have recently shown that insertion of cloned cDNA for the canine histamine H_2 -receptor into COS cells results in expression of a receptor that responds to histamine by activating both the cAMP and InsP_3 pathways [11]. On the other hand, it has been known for several years that addition of InsP_3 to permeabilized cells releases only 30–50 percent of calcium in non-mitochondrial calcium storage pools. These data and other findings, including the detection of calcium oscillations in single cells, have led to several refinements in the hypothesis that InsP_3 -dependent mechanisms are solely responsible for agonist-dependent release of $[\text{Ca}^{2+}]_i$ [38,39]. A currently popular model suggests that an interaction between InsP_3 -sensitive and -insensitive pools leads to oscillations in the calcium signal. Calcium released from an InsP_3 -sensitive pool activates calcium-induced calcium release (CICR) from InsP_3 -insensitive store(s). This concept is based on experiments with skeletal and cardiac muscle, in which a sudden increase in $[\text{Ca}^{2+}]_i$ causes further calcium release. In muscle, CICR is enhanced by low concentrations of the plant alkaloid, ryanodine, and inhibited by high ryanodine concentrations. CICR is also enhanced by caffeine, which activates a calcium channel in the sarcoplasmic reticulum [19,24]. Recently it has been suggested that ryanodine and/or caffeine alter calcium metabolism in pancreatic and salivary acinar cells and adrenal chromaffin cells [20–23,40]. In another study, however, caffeine had a negligible effect on calcium release in permeabilized pancreatic acinar cells [41]. These latter results are similar to our findings in parietal cells, which suggest that there are distinct differences in the mechanisms associated with intracellular calcium release in parietal cells as compared to muscle and neuron and perhaps acinar cells. As yet, the presence of ryanodine receptors that represent another class of intracellular calcium channel in parietal cells in addition to the InsP_3 -receptor-operated channel cannot be completely ruled out because a recent study has detected a widespread distribution of a ryanodine receptor distinct from the muscle subtype [42]. Since oscillations in parietal cell $[\text{Ca}^{2+}]_i$ appear to occur in less than 30 percent of cells in the population ([17] and present study), however, it appears that, at least in parietal cells, calcium oscillations are unnecessary for activation of HCl secretion. Whether calcium oscillations are required for some other parietal cell function or for chief cell pepsinogen secretion remains to be determined.

The single-cell recordings in this study suggest that calcium entry into parietal cells may be differentially regulated by histamine, gastrin, and carbachol because $[\text{Ca}^{2+}]_i$ is sustained at different levels following the initial spike. Several models have been proposed to explain how release of calcium from intracellular storage pools might be linked to activation of calcium influx. For example, Putney recently revised his capacitive calcium entry model, which suggested a direct morphological link between agonist-sensitive intracellular calcium pools and calcium entry, proposing now that calcium uptake and release sites are morphologically separate, with the calcium

release site being closely coupled to the plasma membrane by means of actin filaments [43,44]. The mechanisms by which agonists increase calcium influx in parietal cells is unknown. Unlike excitable cells, which possess both voltage- and receptor-operated calcium channels, the gene families of which have now been identified and cloned, non-excitable cells, including the parietal cell, do not appear to possess such calcium entry mechanisms [1,3,39,45,46]. In several cell types, experiments with Mn have been interpreted to reflect agonist-dependent activation of plasma membrane divalent cation channels [30,31,39]; however, the reliability of measurements that rely on Mn quench of fura-2 fluorescence has recently been challenged [47]. We agree that such experimental protocols can potentially generate a number of artifacts and provide data to show that, when calcium-insensitive wavelengths are utilized for fura-2 measurements, there is no evidence for agonist-stimulated Mn entry in parietal cells. Furthermore, in single-cell experiments (not shown), there was no evidence for Mn influx into parietal cells under either resting or stimulated conditions. These results are in contrast to observations in hepatocytes, where a Mn entry pathway appears to be present in unstimulated cells [47]. Since we were unable to detect significant Mn influx in stimulated or unstimulated parietal cells, we believe it is unlikely that calcium entry in this cell type occurs via agonist-induced opening of plasma membrane-associated divalent cation channels.

In previously published work, it was shown that L-type calcium channel blockers such as verapamil, nifedipine, and nicardipine do not antagonize calcium entry into parietal cells when administered at concentrations that potentially inhibit voltage-dependent calcium entry into excitable cells. Verapamil and nicardipine are capable of inhibiting AP accumulation when administered at supramaximal concentrations; however, nifedipine, which appears to be more specific than verapamil and nicardipine in that it does not inhibit agonist-receptor binding or exhibit local anesthetic-like activity, has not been found to inhibit AP accumulation at concentrations as high as 10 μ M [45]. On the basis of present and previous observations, we hypothesize that agonist-stimulated calcium influx into parietal cells and perhaps other non-excitable secretory cell types may involve an unidentified calcium channel subtype with unique molecular characteristics or, perhaps, a cation or anion exchange mechanism. The differences in the sustained calcium response to histamine, carbachol, and gastrin suggest that the calcium entry mechanism is either differentially regulated by specific second messengers or that more than one calcium entry pathway is involved. Since calcium influx is required for the maintenance of parietal cell HCl secretion in response to calcium-dependent agonists such as carbachol, the elucidation of the factors involved in this process should allow significant advances in the field.

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