Receptor-operated Ca^{2+} channels in gastric parietal cells: gastrin and carbachol induce Ca^{2+} influx in depleting intracellular Ca^{2+} stores

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The mechanism whereby gastrin-type receptor and muscarinic M3-type receptor regulate free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was studied in rabbit gastric parietal cells stimulated by either gastrin or carbachol. Both agonists induced a biphasic $[\text{Ca}^{2+}]_i$ response: a transient $[\text{Ca}^{2+}]_i$ rise, followed by a sustained steady state depending on extracellular Ca^{2+} . Gastrin and carbachol also caused a rapid and transient increase in Mn^{2+} influx (a tracer for bivalent-cation entry). Pre-stimulation of cells with one agonist drastically decreased both $[\text{Ca}^{2+}]_i$ increase and Mn^{2+} influx induced by the other. Neither diltiazem nor pertussis-toxin treatment had any effect on agonist-stimulated Mn^{2+} entry. Thapsigargin, a Ca^{2+} -pump inhibitor, induced a biphasic $[\text{Ca}^{2+}]_i$ increase, and enhanced the rate of Mn^{2+} entry. Pre-

incubation of cells with thapsigargin inhibits the $[\text{Ca}^{2+}]_i$ increase as well as Mn^{2+} entry stimulated by gastrin or by carbachol. Thapsigargin induced a weak but significant increase in $\text{Ins}(1,4,5)\text{P}_3$ content, but this agent had no effect on the agonist-evoked $\text{Ins}(1,4,5)\text{P}_3$ response. In permeabilized parietal cells, $\text{Ins}(1,4,5)\text{P}_3$ and caffeine caused an immediate Ca^{2+} release from intracellular pools, followed by a reloading of Ca^{2+} pools which can be prevented in the presence of thapsigargin. We conclude that (i) gastrin and carbachol mobilize common Ca^{2+} intracellular stores, (ii) Ca^{2+} permeability secondary to receptor activation involves neither a voltage-sensitive Ca^{2+} channel nor a GTP-binding protein from the G_i family, and (iii) agonists regulate common Ca^{2+} channels in depleting intracellular Ca^{2+} stores.

INTRODUCTION

Changes in the cytosolic free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) play a crucial role in the stimulus-secretion coupling in a variety of tissues [1]. Agonists that elicit phosphoinositide (PtdInsPs) hydrolysis are known to induce a biphasic $[\text{Ca}^{2+}]_i$ response: an initial spike obtained primarily from the $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} from non-mitochondrial intracellular stores, followed by a plateau phase, owing to a Ca^{2+} influx from extracellular medium [2,3]. Although the mechanism by which agonists induce Ca^{2+} release from intracellular stores is well documented, the pathway whereby receptor activation induces Ca^{2+} entry remains obscure [4].

In many non-excitable cells, Ca^{2+} influx observed after receptor-induced PtdInsPs hydrolysis occurs through receptor-operated voltage-independent Ca^{2+} channels [4,5]. Such a Ca^{2+} influx has been proposed to be regulated by second messengers, including $\text{Ins}(1,4,5)\text{P}_3$ [6,7] $\text{Ins}(1,3,4,5)\text{P}_4$ [8–10] and GTP-binding proteins from the G_i family [11,12]. On the other hand, Putney [13] has proposed an alternative route, known as ‘capacitance entry’, in which Ca^{2+} influx results from emptying of non-mitochondrial intracellular pools. Thapsigargin, a tumour-promoting sesquiterpene lactone, has been shown to deplete the non-mitochondrial Ca^{2+} stores in inhibiting the Ca^{2+} pumps responsible for the refilling of these stores [14,15]. So thapsigargin is a valuable tool with which to investigate the relationship between intracellular Ca^{2+} stores and Ca^{2+} entry and allows confirmation of the model proposed by Putney in some cell types, including parotid acinar cells [14], T lymphocytes [16] or adrenal glomerulosa cells [17]. Nevertheless, in chromaffin cells [18], platelets [19], hepatocytes [20] or human fibroblasts [21], Ca^{2+} entry subsequent to receptor activation can be dissociated from intracellular pool depletion, in disagreement with the proposed capacitative model. Therefore, several Ca^{2+} influx pathways can be postulated in one cell type.

In gastric parietal cells, the mechanism whereby receptor-induced PtdInsPs hydrolysis regulates Ca^{2+} influx remains elusive. Gastrin and acetylcholine are known to enhance the acid-secretory activity of parietal cells through activation of, respectively, a ‘gastrin-type’ [22] receptor and a muscarinic M3-type receptor [23–25], both coupled to PtdInsPs hydrolysis [22, 24–27]. The rise in $[\text{Ca}^{2+}]_i$ following activation of these receptors is biphasic [28–31], as observed in other cell systems. Ca^{2+} influx may play an important role, at least for acetylcholine, to ensure the full secretory response [31,32]. A previous report suggested the involvement of a voltage-sensitive Ca^{2+} channel, since L-type Ca^{2+} channel blockers were shown to inhibit carbachol-induced acid secretion ([32]; A. Léonard, unpublished work); but such an effect appeared not to be specific, as these Ca^{2+} blockers inhibited in the meantime the acid-secretory response induced by histamine, a secretagogue devoid of effect on Ca^{2+} entry [32]. More recently, Negulescu and Machen [30] reported the absence of effect of voltage-sensitive Ca^{2+} -channel blockers on the regulation of $[\text{Ca}^{2+}]_i$ by carbachol; in addition, they obtained evidence of the ability of parietal cells to reload Ca^{2+} stores in Ca^{2+} -free medium [33], in contrast with Putney’s [13] capacitative model.

On the other hand, the existence of second-messenger-operated Ca^{2+} channels cannot be precluded in these secretory cells, since an accumulation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ occurred after stimulation by agonists [31,34]. Furthermore, in recent reports, we have suggested the existence of a ‘ G_i -like protein’ that could be involved in the action of gastrin on parietal cells [35,36]. Thus the mechanism for receptor-operated Ca^{2+} entry has to be clarified in gastric parietal cells.

In many non-excitable cells, Mn^{2+} competes with Ca^{2+} for entering the cell via receptor-operated Ca^{2+} channels. Hallam and co-workers [37] have developed a new technique which allows the continuous measurement of Ca^{2+} entry by means of the ability of Mn^{2+} to bind to fluorescent Ca^{2+} indicators with a higher affinity than does Ca^{2+} , and in turn to quench their

Abbreviations used: $[\text{Ca}^{2+}]_i$, free intracellular Ca^{2+} concentration; PtdInsPs , phosphoinositides.

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fluorescence strongly. Therefore, the agonist-stimulated decrease in fluorescence will reflect the stimulation of Mn^{2+} entry via Ca^{2+} channels.

The aim of the present work was to study the regulation of $[Ca^{2+}]_i$ in rabbit gastric parietal cells stimulated by gastrin and carbachol; furthermore, using Mn^{2+} as a tracer for bivalent-cation entry, we investigated the relationship between agonist-induced Ca^{2+} influx and depletion of intracellular Ca^{2+} stores induced by thapsigargin.

EXPERIMENTAL

Unsulphated gastrin ([Ahx11]-HG-17) was a gift from Professor E. Wünsch and Professor L. Moroder (Max Planck Institut für Biochemie, München, Germany). Devazepide and L365,260 were obtained from Dr. Paul Anderson (Merck Sharp & Dohme, West Point, U.S.A.). Carbachol, atropine, thapsigargin, pertussis toxin, saponin, EGTA, $Ins(1,4,5)P_3$, caffeine, phosphocreatine and creatine kinase were from Sigma (St. Louis, MO, U.S.A.). Fura-2/AM and Fura-2 were from Molecular Probes (U.S.A.). $Ins(1,4,5)P_3$ radioreceptor assay kit was from Dupont de Nemours – NEN Division (France). Collagenase was from Serva (Heidelberg, Germany). Nycodenz was from Accurate Chemicals (U.S.A.). Standard medium comprised 116 mM NaCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4 , 1.8 mM $CaCl_2$, 5.5 mM glucose, 10 mM Hepes and 0.1% BSA, pH 7.4.

Preparation of isolated parietal cells

Cell isolation from rabbit gastric mucosa was carried out by the collagenase/EDTA procedure as previously described [34]. Cell separation was performed by counterflow centrifugation with a Beckman elutriator rotor JE6-B. The elutriation fraction containing 60–75% of parietal cells was layered on to a Nycodenz gradient ($d = 1.04$ – 1.08). Top fractions, which contained 85–95% of parietal cells, ($\geq 90\%$ viability) were collected and used for subsequent studies.

Pretreatment of parietal cells with pertussis toxin

Cells (5×10^6 cells/ml) were incubated for 2 h at $37^\circ C$ in standard medium with or without pertussis toxin (200 ng/ml) under continuous gassing (O_2/CO_2 , 19:1). Cells were then centrifuged (200 g, 5 min) and resuspended in standard medium (1.5×10^6 cells/ml).

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

The fluorescent probe Fura-2/AM was used to assess $[Ca^{2+}]_i$ as described by Léonard et al. [31]. Briefly, cells were incubated in standard medium in the presence of Fura-2/AM (2 μM) for 20 min at $37^\circ C$. Cells were then washed by centrifugation (200 g, 5 min) and cell pellet was resuspended in standard medium (1.5×10^6 cells/ml). In some experiments Ca^{2+} was omitted and 0.5 mM EGTA was added in the medium 30 s before addition of the agent. Fluorescence measurements were performed with a Kontron SFM-25 spectrofluorimeter. The excitation and emission wavelengths were 340 and 510 nm respectively. Cells were subsequently lysed by adding Triton X-100 (final concn. 0.3%) to obtain the signal of the Ca^{2+} -saturated dye (F_{max}). F_{min} for the Ca^{2+} -free form of the dye was recorded by adding 10 mM EGTA, pH 8.5. $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where $K_d = 224$ nM.

Mn^{2+} -influx measurement

Mn^{2+} entry was measured by the method described by Hallam and co-workers [37]. Fluorescence was recorded for Fura-2-loaded parietal cells after addition of 500 μM $MnCl_2$. In some experiments Ca^{2+} was omitted from the medium. The excitation and emission wavelengths used were 360 nm (corresponding to the isostilbic point of Fura-2 for Ca^{2+}) and 510 nm respectively. Under these conditions, the fluorescence signal is independent of $[Ca^{2+}]$ within the cells. The rate of Mn^{2+} entry was calculated from the initial rate of the fluorescence change occurring after agent addition and is expressed as arbitrary units/min.

Measurement of $Ins(1,4,5)P_3$ content

$Ins(1,4,5)P_3$ content was determined by a radioreceptor assay as described previously [27]. Cells (4×10^6 cells/ml) were equilibrated for 5 min at $37^\circ C$ in standard medium and then stimulated with or without agent to be tested for various periods of time. The reaction was stopped by adding $HClO_4$ (5%, w/v) and the supernatants were neutralized with 3 M KOH. Portions (100 μl) of supernatants were incubated for 1 h at $4^\circ C$ with $[^3H]Ins(1,4,5)P_3$ (~ 7 nCi) and with a specific $Ins(1,4,5)P_3$ -binding protein. After centrifugation (12000 g, 3 min), the supernatant was removed and the pellet assayed for radioactivity content. $Ins(1,4,5)P_3$ content in the sample was calculated from a standard curve with authentic $Ins(1,4,5)P_3$.

Measurement of Ca^{2+} release from permeabilized cells

Parietal cells were permeabilized as described previously [35]. Briefly, cells (10×10^6 /ml) were washed twice with permeabilization medium containing 20 mM NaCl, 150 mM KCl, 5 mM $MgSO_4$, 0.2 mM KH_2PO_4 , 0.8 mM K_2HPO_4 , 10 mM Hepes, 4 mM ATP, 5 mM phosphocreatine and 100 units of creatine kinase (pH 7.2). Cells were incubated for 10 min with 40 μg of saponin/ml at $37^\circ C$ and then diluted to a final concentration of 2.5×10^6 cells/ml with permeabilization medium. $[Ca^{2+}]_i$ measurement was performed as described above for $[Ca^{2+}]_i$ measurement after adding Fura-2 (2 μM) to the cell suspension. $CaCl_2$ (2 mM final concn.) was subsequently added to obtain the signal of the Ca^{2+} -saturated dye (F_{max}). F_{min} for the Ca^{2+} free form of the dye was recorded by adding EGTA (10 mM, pH 8.5).

Statistical analysis

Values are means $\pm$ S.E.M. from 3–10 separate experiments. Data were analysed for statistical significance by Student's *t* test.

RESULTS

Change in $[Ca^{2+}]_i$ following muscarinic- and gastrin-receptor activation

Gastrin induced a rapid rise in $[Ca^{2+}]_i$ from 218 ± 8 to 285 ± 15 nM in Fura-2-loaded parietal cells. After this transient response, $[Ca^{2+}]_i$ returned to a small but significant steady-state value corresponding to 16 ± 5 nM above basal level; this plateau phase can be abolished in the absence of extracellular Ca^{2+} (result not shown). The change in $[Ca^{2+}]_i$ can be directly related to activation of a 'gastrin-type' receptor, since L365,260, a selective gastrin/CCK-B-type receptor antagonist [22,38], was 70–100-fold more potent than devazepide, a selective antagonist of CCK-A-type receptor [39], in inhibiting the rise in $[Ca^{2+}]_i$ induced by gastrin (IC_{50} values were 2.3 ± 0.4 nM for L365,260 and 187 ± 35 nM for devazepide; results not shown). Carbachol also induced biphasic

Figure 1 Effect of gastrin and carbachol on $[\text{Ca}^{2+}]_i$ in gastric parietal cells

Cells were incubated for 20 min at 37 °C with 2 μM Fura-2/AM. After washing, cell fluorescence (340 nm excitation, 510 nm emission) was recorded as a function of time. Cells were preincubated for 2 h at 37 °C without (a) or with (b) pertussis toxin (200 ng/ml) before loading with Fura-2 AM and then stimulated with gastrin (HG-17; 0.1 μM). Cells were preincubated for 2 h at 37 °C without (c) or with (d) pertussis toxin (200 ng/ml) before loading with Fura-2 AM and then stimulated with carbachol (100 μM). $[\text{Ca}^{2+}]_i$ was expressed in nM as indicated in the Experimental section. Data are shown from one of five similar experiments.

Figure 2 Effect of gastrin and carbachol on Mn^{2+} permeability in gastric parietal cells

Cells were loaded with 2 μM Fura-2/AM for 20 min at 37 °C. After washing, cell fluorescence (360 nm excitation, 510 nm emission) was recorded as a function of time; Mn^{2+} (500 μM) was added in the medium before agonist addition; (a) cells were stimulated with gastrin (HG-17; 0.1 μM), then carbachol (CCh; 100 μM) was added; (b) cells were stimulated with carbachol (100 μM), then HG-17 (0.1 μM) was added. Cell fluorescence was recorded and expressed as arbitrary units. Data are shown from one of four similar experiments.

kinetics in the $[\text{Ca}^{2+}]_i$ response in this cell type: a rapid increase up to 392 ± 27 nM, followed by a plateau level depending on extracellular Ca^{2+} , and corresponding to 37 ± 8 nM above basal level. This $[\text{Ca}^{2+}]_i$ response has been related to muscarinic M3-type receptor activation [24].

Chew and Brown [28] previously demonstrated that carbachol and CCK-8 increased parietal-cell $[\text{Ca}^{2+}]_i$ initially by release of Ca^{2+} from the same intracellular store. In the same way, when cells were pre-stimulated with gastrin, the rapid $[\text{Ca}^{2+}]_i$ rise induced by carbachol was inhibited by 30–50 % as compared with the response induced by carbachol alone; similarly, the gastrin-induced $[\text{Ca}^{2+}]_i$ rise was inhibited when cells were pre-stimulated with carbachol, compared with the response induced by gastrin alone (results not shown). Moreover, even if pertussis-toxin treatment of cells affected gastrin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ production, this toxin did not affect the gastrin- or carbachol-induced $[\text{Ca}^{2+}]_i$ increase (Figure 1).

Ca^{2+} -mobilizing agonists stimulate Mn^{2+} entry in gastric parietal cells

To investigate the mechanism whereby gastrin and carbachol stimulate Ca^{2+} influx, we studied the ability of these agonists to induce Mn^{2+} entry. As shown in Figure 2(a), addition of Mn^{2+} (500 μM) into the suspension of Fura-2-loaded cells led to an immediate fall in the fluorescence intensity (< 10 % of the maximal fluorescence). This decrease could be attributed to the binding of Mn^{2+} to the dye present in the extracellular medium. Then, a slight decrease in fluorescence occurred, presumably owing to a slow intake of Mn^{2+} into parietal cells, corresponding to 1.65 ± 0.15 fluorescence units/min. Gastrin or carbachol markedly enhanced the rate of Fura-2 fluorescence quenching (Figure 2), consistent with a stimulation of Mn^{2+} influx into the cells. This initial decrease after agonist addition corresponded to 36 ± 1.75 units/min for gastrin and 52 ± 2.2 units/min for carbachol. This rapid decrease was transient and occurred within the first seconds after agonist addition, then the fluorescence quenching rate returned to the basal value after 30–60 s stimulation. This accelerated Mn^{2+} entry due to gastrin or carbachol stimulation was inhibited in the presence of gastrin- or muscarinic-receptor antagonists (Table 1).

Carbachol-stimulated Mn^{2+} influx was drastically decreased when cells were pre-stimulated with gastrin (Figure 2a); similarly, gastrin-induced Mn^{2+} influx was inhibited when cells were pre-stimulated with carbachol (Figure 2b).

As shown in Table 1, removal of Ca^{2+} from the extracellular medium or addition of diltiazem, a L-type Ca^{2+} -channel blocker, did not affect Mn^{2+} influx caused by agonist; furthermore, a pretreatment of cells with pertussis toxin did not change this Mn^{2+} influx (Table 1).

Relationship between thapsigargin- and agonist-induced Ca^{2+} mobilization

Thapsigargin has been described to affect $[\text{Ca}^{2+}]_i$ by inhibiting Ca^{2+} pumps, leading to emptying of the Ca^{2+} store in several tissues [14,15]. As shown by Chew and Petropoulos [40], in parietal cells thapsigargin induced biphasic kinetics in the $[\text{Ca}^{2+}]_i$ response: an increase up to 383 ± 35 nM, followed by a sustained steady-state corresponding to 75 ± 17 nM above the basal value; this plateau level was abolished in a Ca^{2+} -free medium (results not shown). Maximal increase was obtained after 30–40 s stimulation with thapsigargin, whereas agonists induced maximal increase in $[\text{Ca}^{2+}]_i$ after 5–10 s.

Thapsigargin pretreatment of cells for more than 3 min led to a

Table 1 Effects of diltiazem, pertussis-toxin (PTx) treatment and various receptor antagonists on gastrin- and carbachol-stimulated Mn^{2+} entry

Cells were loaded with 2 μM Fura-2/AM for 20 min at 37 °C. After washing, cell fluorescence (360 nm excitation, 510 nm emission) was recorded as a function of time. Mn^{2+} (500 μM) was then added in the medium; agents to be tested were added 15 s before gastrin (HG-17; 0.1 μM) or carbachol (100 μM) addition. Mn^{2+} entry rate was calculated from the initial fluorescence quenching rate and expressed as arbitrary units/min. Basal Mn^{2+} rate entry was 1.65 ± 0.15 units/min. Data are means $\pm$ S.E.M. from 3–5 separate experiments (n.d., not determined). Significance of differences from data obtained with agonist-stimulated cells, as mentioned in the Experimental section: * $P < 0.01$, ** $P < 0.001$.

	Mn^{2+} entry rate	
	Gastrin	Carbachol
Control cells	36.00 ± 1.75	52.00 ± 2.20
+ 0.5 μM Atropine	n.d.	$6.00 \pm 0.75^{**}$
+ 0.1 μM L365,260	$2.19 \pm 0.45^{**}$	n.d.
+ 0.1 μM devazepide	$23.05 \pm 3.70^*$	n.d.
+ 0.1 mM Diltiazem	32.20 ± 3.70	53.40 ± 5.70
PTx-treated cells	42.00 ± 4.10	59.00 ± 3.20
0 extracellular $[Ca^{2+}]$	34.20 ± 3.50	49.50 ± 5.40

Figure 3 Effect of thapsigargin (T) on $[Ca^{2+}]_i$ in unstimulated or agonist-stimulated cells

Cells were incubated for 20 min at 37 °C with 2 μM Fura-2/AM. After washing, cell fluorescence (340 nm excitation, 510 nm emission) was recorded as a function of time; (a) cells were stimulated with thapsigargin, then gastrin (HG-17; 0.1 μM) was added; (b) cells were stimulated with thapsigargin, then carbachol (CCh; 100 μM) was added; (c) cells were stimulated with HG-17, then thapsigargin was added. $[Ca^{2+}]_i$ was expressed in nM as indicated in the Experimental section. Data are shown from one of four similar experiments.

total inhibition of either gastrin- or carbachol-induced $[Ca^{2+}]_i$ increase (Figures 3a and 3b). Furthermore, the $[Ca^{2+}]_i$ response stimulated by thapsigargin was decreased when the cells were prestimulated with gastrin (Figure 3c).

Relationship between thapsigargin- and agonist-induced Mn^{2+} entry

As shown in Figure 4, in the presence of Mn^{2+} thapsigargin

Figure 4 Effect of thapsigargin on Mn^{2+} influx in unstimulated or agonist-stimulated cells

Cells were loaded with 2 μM Fura-2/AM for 20 min at 37 °C. After washing, cell fluorescence (360 nm excitation, 510 nm emission) was recorded as a function of time; Mn^{2+} (500 μM) was added in the medium before agonist addition. (a) Cells were stimulated with thapsigargin (T) (2 μM) (continuous line) or not (broken line), then gastrin (HG-17; 0.1 μM) was added; (b) cells were stimulated with thapsigargin (continuous line) or not (broken line), then carbachol (CCh; 100 μM) was added. Cell fluorescence was recorded and expressed as arbitrary units. Data are shown from one of four similar experiments.

increased the rate of fluorescence quenching within the first seconds of stimulation, but the initial rate of quenching (13.5 ± 1.1 arbitrary units/min) was lower than that obtained with agonists. Furthermore, the initial rate of Mn^{2+} entry appeared to be slower than that observed with agonists: basal Mn^{2+} entry was recovered 1.5–2 min after thapsigargin addition. Pre-stimulation of cells with thapsigargin for 3 min abolished the Mn^{2+} entry induced by gastrin or carbachol (Figures 4a and 4b).

Effect of thapsigargin on agonist-stimulated $Ins(1,4,5)P_3$ accumulation

To evaluate whether thapsigargin interacts with gastrin or muscarinic receptors, the effect of this agent on $Ins(1,4,5)P_3$ production was studied. Thapsigargin alone led to a weak but significant increase in $Ins(1,4,5)P_3$ content within 5–10 min of stimulation: 7.8 ± 0.4 pmol/500 000 cells after 5 min stimulation, compared with a basal value of 5.8 ± 0.3 pmol/500 000 cells (Figure 5). Gastrin induced a biphasic increase in $Ins(1,4,5)P_3$ content (Figure 6a), and preincubation of cells for 5 min with thapsigargin did not modify this gastrin-stimulated $Ins(1,4,5)P_3$

Figure 5 Time course of $\text{Ins}(1,4,5)\text{P}_3$ accumulation in thapsigargin-stimulated parietal cells

Parietal cells were incubated with ($\square$) or without ($\triangle$) thapsigargin ($2 \mu\text{M}$) at the indicated times; $\text{Ins}(1,4,5)\text{P}_3$ was quantified as described in the Experimental section. Results are expressed as pmol/500 000 cells and are means from 5 separate experiments. Stimulated $\text{Ins}(1,4,5)\text{P}_3$ levels are statistically different from basal $\text{Ins}(1,4,5)\text{P}_3$ accumulation at $P < 0.01$.

production (Figure 6b). Carbachol also induced a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ mass which was maximal by 30 s, then slowly declined after 5 min (Figure 7a); as observed with gastrin, pretreatment of cells with thapsigargin for 5 min had no effect on the $\text{Ins}(1,4,5)\text{P}_3$ rise caused by carbachol (Figure 7b).

Effect of thapsigargin on refilling of Ca^{2+} stores in permeabilized parietal cells

In permeabilized parietal cells, $\text{Ins}(1,4,5)\text{P}_3$ ($100 \mu\text{M}$) induced a large and immediate Ca^{2+} release from intracellular pools (Figure 8a). Caffeine (10 mM), known to release Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -insensitive/ Ca^{2+} -sensitive Ca^{2+} stores, also provoked a rapid increase in $[\text{Ca}^{2+}]_i$ (Figure 8b). $[\text{Ca}^{2+}]_i$ slowly returned within 10 min to the basal level after depletion of Ca^{2+} stores caused by caffeine plus $\text{Ins}(1,4,5)\text{P}_3$ (results not shown). Thapsigargin addition after depletion of Ca^{2+} stores by either agent prevented this return to basal level (Figures 8a and 8b).

DISCUSSION

The aim of this work was to study the regulation of $[\text{Ca}^{2+}]_i$ in gastric parietal cells stimulated by gastrin or carbachol, and to investigate the mechanism whereby the receptors of these agents operate Ca^{2+} channels in parietal cells.

Gastrin and carbachol mobilize common intracellular Ca^{2+} stores

Gastrin and carbachol are known to increase acid-secretory activity of parietal cells, primarily by stimulating phosphoinositide breakdown [22,24–27]. Although carbachol-regulated $[\text{Ca}^{2+}]_i$ has been studied in these cells [28,30,31,33], less is known about the $[\text{Ca}^{2+}]_i$ response secondary to 'gastrin-type' receptor activation. Stimulation of both gastrin-type receptor and M3-type receptor induces a biphasic $[\text{Ca}^{2+}]_i$ increase, as described in other cell systems: a rapid $[\text{Ca}^{2+}]_i$ rise owing to discharge of Ca^{2+} stores, followed by a Ca^{2+} influx from the extracellular medium. The fact that a pre-stimulation of cells with one agonist led to a decrease in the $[\text{Ca}^{2+}]_i$ rise induced by the other suggests that

Figure 6 Effect of thapsigargin on $\text{Ins}(1,4,5)\text{P}_3$ accumulation in gastrin-stimulated parietal cells

(a) Cells were incubated without ($\triangle$) or with ($\blacktriangle$) gastrin (10 nM) at indicated times in the absence of thapsigargin. (b) Cells were preincubated with thapsigargin for 5 min at 37°C , then cells were incubated without ($\square$) or with ($\blacksquare$) gastrin (10 nM) at indicated times. $\text{Ins}(1,4,5)\text{P}_3$ was quantified as described in the Experimental section. Results are expressed as pmol/500 000 cells. Data are shown from one of three similar experiments.

gastrin and carbachol may mobilize Ca^{2+} from common Ca^{2+} stores in these cells. As described in other cell systems [1,2], the rapid $[\text{Ca}^{2+}]_i$ increase induced by gastrin or by carbachol in parietal cells may be initiated by the $\text{Ins}(1,4,5)\text{P}_3$ -elicited Ca^{2+} release, since (i) agonists caused a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ content within the first seconds of stimulation [31,36], and (ii) $\text{Ins}(1,4,5)\text{P}_3$ induced a large $[\text{Ca}^{2+}]_i$ increase in permeabilized cells ([41]; the present study).

Gastrin receptor and muscarinic receptor regulate common Ca^{2+} channels

To characterize the mechanism whereby gastrin and carbachol induced Ca^{2+} entry, we have studied Mn^{2+} influx into Fura-2-loaded parietal cells. This technique provided evidence for receptor-operated Mn^{2+} -sensitive Ca^{2+} channels. In parietal cells, selective antagonists for gastrin-type receptor and muscarinic receptor inhibit in a parallel fashion $[\text{Ca}^{2+}]_i$ increase and Mn^{2+} entry, suggesting that discharge of the intracellular Ca^{2+} pool and Ca^{2+} influx may be regulated through the same gastrin

Figure 7 Effect of thapsigargin on Ins(1,4,5)P₃ accumulation in carbachol-stimulated parietal cells

(a) Cells were incubated without (Δ) or with (▲) carbachol (100 μM) at the indicated times in the absence of thapsigargin. (b) Cells were preincubated with thapsigargin for 5 min at 37 °C, and then incubated without (□) or with (■) carbachol (100 μM) at the indicated times. Ins(1,4,5)P₃ was quantified as described in the Experimental section. Results are expressed as pmol/500 000 cells. Data are shown from one of three similar experiments.

Figure 8 Effect of thapsigargin on Ins(1,4,5)P₃- or caffeine-induced [Ca²⁺]_i rise in permeabilized parietal cells

Saponin-permeabilized cells were incubated at 37 °C, then Fura-2 (2 μM) was added; cell fluorescence (340 nm excitation, 510 nm emission) was recorded as a function of time. Cells were stimulated with Ins(1,4,5)P₃ (a) or with caffeine (c; 10 mM) (b), then thapsigargin (T) was added. [Ca²⁺]_i was expressed in nM as indicated in the Experimental section. Data are shown from one of three similar experiments.

receptor or the same muscarinic receptor. Furthermore, bivalent entry induced by gastrin and by carbachol seems to occur through common Ca²⁺ channels, since (i) agonist-stimulated Mn²⁺ influx responses were not additive and (ii) pre-stimulation of cells with one agonist drastically decreased the Mn²⁺ influx induced by the other.

The mechanism by which these receptors operate Ca²⁺ channels in gastric parietal cells was then investigated. In agreement with a previous report [33], Ca²⁺ influx seems not to occur through voltage-sensitive Ca²⁺ channels, since diltiazem had no effect on agonist-induced Mn²⁺ entry. Moreover, it is unlikely that this process involves a GTP-binding protein of the G_i family, as pertussis-toxin treatment of cells affected neither the [Ca²⁺]_i response nor the Mn²⁺ entry stimulated by gastrin or carbachol under the conditions in which this toxin affected gastrin-stimulated phospholipase C activation [35,36] and the adenylate cyclase-G_i subunit of the prostaglandin receptor [42]. An alternative explanation would be that Ins(1,4,5)P₃ induces Ca²⁺-channel opening, as shown in T lymphocytes [6]; but in our case the withdrawal of the extracellular Ca²⁺ led to a drastic decrease in Ins(1,4,5)P₃ content of gastrin [36]- or carbachol (results not shown)-stimulated parietal cells, whereas agonist-stimulated Mn²⁺ entry was not modified. Taken together, these observations suggest that Ins(1,4,5)P₃ does not play a direct role in opening of the Ca²⁺ channel induced by agonists.

Thapsigargin inhibits the refilling of Ins(1,4,5)P₃-sensitive and caffeine-sensitive Ca²⁺ stores in gastric parietal cells

A possible relationship between the emptying of intracellular Ca²⁺ pools and receptor-operated Ca²⁺ channels was investigated in gastric parietal cells. First, Ca²⁺ stores were identified in permeabilized cells by their sensitivity to Ins(1,4,5)P₃ and to caffeine, an agonist of voltage-dependent Ca²⁺ channels [1,2]. Our results are consistent with the existence of at least two distinct Ca²⁺ stores in parietal cells, since caffeine can further release Ca²⁺ in permeabilized cells even after depletion of Ca²⁺ stores provoked by a maximal concentration of Ins(1,4,5)P₃. These results are in agreement with a previous report [41], which showed that Ins(1,4,5)P₃ can partially release Ca²⁺ from intracellular Ca²⁺ stores in permeabilized parietal cells. Moreover, Tsunoda et al. [43] demonstrated that in parietal cells gastrin can induce Ca²⁺ waves propagating within the cytosol, consistent with the existence of two different Ca²⁺ stores in this cell type.

Thapsigargin inhibits the Ca²⁺-ATPase that is responsible for refilling of Ca²⁺ pools in several cell types [14,15]; therefore, this agent is a valuable tool with which to study the relationship between emptying of Ca²⁺ stores and Ca²⁺ influx. As already reported, thapsigargin induced biphasic kinetics of the [Ca²⁺]_i response in gastric parietal cells [40], i.e. a rapid [Ca²⁺]_i increase followed by a Ca²⁺ influx from extracellular medium. Since thapsigargin had no effect on the Ins(1,4,5)P₃ production induced either by gastrin or by carbachol, this [Ca²⁺]_i response may not be due to an interaction of this lactone with these Ca²⁺-mobilizing receptors. As observed in parotid acinar cells [14], thapsigargin induced a [Ca²⁺]_i increase in permeabilized parietal cells, consistent with an inhibition of refilling of Ca²⁺ pools (results not shown). Furthermore, thapsigargin can prevent the reloading of Ca²⁺ stores occurring after Ins(1,4,5)P₃- or caffeine-induced depletion of Ca²⁺ stores. Thus, in parietal cells, this agent must act by inhibiting Ca²⁺-ATPases regulating Ca²⁺ stores reloading.

It can be noticed that, in contrast with several cell systems [14,15], in gastric parietal cells thapsigargin induced a weak but significant increase in Ins(1,4,5)P₃ cellular content as well as an increase in [³H]inositol phosphates accumulation in [³H]inositol-

prelabelled cells (results not shown); this effect could be explained by the PtdInsPs hydrolysis caused by a Ca^{2+} -induced increase in phospholipase C activity, since a Ca^{2+} ionophore can enhance PtdInsPs breakdown in this cell type [36]. However, the observed Ins(1,4,5) P_3 content increase may not be responsible for the thapsigargin-stimulated $[\text{Ca}^{2+}]_i$ response, since (i) thapsigargin alone induced a weak Ins(1,4,5) P_3 accumulation, and (ii) the time course of $[\text{Ca}^{2+}]_i$ rise and of Mn^{2+} entry fit in with an inhibition of Ca^{2+} reloading rather than with a discharge of Ca^{2+} stores caused by Ins(1,4,5) P_3 .

Ca^{2+} influx can occur secondary to discharge of Ca^{2+} pools

Ca^{2+} influx induced by thapsigargin was compared with Ca^{2+} influx induced by agonists. First, the capacitative model proposed by Putney [13] can occur in gastric parietal cells: Ca^{2+} influx can be triggered by emptying of Ca^{2+} pools by thapsigargin, as shown by Mn^{2+} -entry measurements. Thapsigargin, gastrin and carbachol induced a $[\text{Ca}^{2+}]_i$ rise by acting on common Ca^{2+} stores, since preincubation of cells with thapsigargin abolished the agonist-stimulated $[\text{Ca}^{2+}]_i$ response without affecting receptor-induced Ins(1,4,5) P_3 production. Moreover, an important piece of information is that emptying Ca^{2+} stores abolished agonist-induced Mn^{2+} entry. Taken together, these results strongly suggest that, in agreement with the capacitative model, gastrin and carbachol induced Ca^{2+} -channel opening in depleting Ca^{2+} stores.

The molecular relationship between emptying of Ca^{2+} pools and Ca^{2+} influx is currently unknown. A physical link between intracellular stores and the plasma membrane has been suggested in some cell types; in this way, a direct coupling between proteins in the plasma membrane and the membrane of Ca^{2+} stores could be expected. An alternative explanation would be the existence of a diffusible second messenger released from the storage organelles, but a recent report suggested the existence of a cytochrome *P*-450 enzyme [44] as a link between intracellular Ca^{2+} stores and plasma-membrane Ca^{2+} channels.

Our observations seem to be in disagreement with an earlier report by Machen and Negulescu [33] who showed that in gastric parietal cells Ca^{2+} stores could, under certain experimental conditions, be refilled from the cytoplasm; however, these results can be consistent with the capacitative model if one considers that extracellular Ca^{2+} enters the cytoplasm before entering the stores, as suggested for parotid acinar cells [14,45] or lacrimal acinar cells [46]. In favour of this hypothesis, Tsunoda et al. [43] reported high $[\text{Ca}^{2+}]_i$ just beneath the plasma membrane, which enlarged after gastrin stimulation; furthermore, bivalent cations, such as Mn^{2+} , enter the cytoplasm in unstimulated cells, the influx rate increasing after depletion of Ca^{2+} stores. However, further experiments are needed to confirm such a hypothesis.

The fact that agonists may regulate Ca^{2+} entry by depleting Ca^{2+} stores does not preclude the existence of other mechanisms involved in the regulation of Ca^{2+} entry in these cells. Thus, a previous report suggested that carbachol, in the presence of a selective antagonist, could induce Ca^{2+} influx without stimulating Ca^{2+} release from intracellular pools [25]. These authors suggested the existence of two muscarinic receptors in gastric parietal cells: one receptor coupled to Ca^{2+} influx, and the other coupled to a phospholipase C and thus inducing Ca^{2+} release from intracellular pools. Furthermore, a recent report showed that preincubation of A9m3 cells with phorbol 12-myristate 13-acetate abolished M3-receptor-stimulated PtdInsPs hydrolysis and Ca^{2+} release from intracellular stores without affecting receptor-operated Ca^{2+} influx [47]. Thus several mechanisms regulating Ca^{2+} entry after

M3-receptor activation could be expected. In this way, several Ca^{2+} -influx pathways have been proposed in platelets [19] or in human fibroblasts [21]: Ca^{2+} channels regulated by emptying of Ca^{2+} stores and a Ca^{2+} channel insensitive to the filling state of these stores. In keeping with this, our results do not preclude the existence of Mn^{2+} -insensitive Ca^{2+} -influx pathways, which could not be observed in our experimental conditions. Note that agonist-activated Ca^{2+} channels do not allow Mn^{2+} to pass in lacrimal acinar cells [46] or in parotid acinar cells [48].

In conclusion, this paper first demonstrates that in gastric parietal cells gastrin-type and muscarinic M3-type receptors can regulate Ca^{2+} influx by depleting intracellular Ca^{2+} stores, in agreement with the capacitative model proposed by Putney [13]. In addition, our results suggest the existence of different types of Ca^{2+} stores, which could play an important role in the regulation of the acid-secretory process of the parietal cell.

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