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Role of Ca^{2+} in H^+ transport by rabbit gastric glands studied with A23187 and BAPTA, an incorporated Ca^{2+} chelator

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The role of Ca^{2+} in stimulation of H^+ gastric secretion by cAMP-dependent and -independent secretagogues was studied in isolated rabbit glands using Ca^{2+} ionophore, A23187, and an intracellular Ca^{2+} chelator (BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) incorporated as its acetoxymethyl ester (BAPTA-AM). Acetylcholine (ACh), tetragastrin (TG), histamine and forskolin induced a transitory increase of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, measured in gastric glands loaded with Ca^{2+}-sensitive dye fura-2, and provoked an acid secretory response evaluated with aminopyrine accumulation ratio (AP ratio). The Ca^{2+}-ionophore A23187 also induced an increase in $[Ca^{2+}]_i$ and in AP ratio. cAMP-dependent secretagogues were more potent stimulants of acid secretion than cAMP-independent secretagogues. cAMP analogue, 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-BR-cAMP) induced an increase in AP ratio without modifying $[Ca^{2+}]_i$. BAPTA-AM (5–25 μM) induced a transient decrease of resting $[Ca^{2+}]_i$ which returned to basal level due to extracellular Ca^{2+} entry. Increases in $[Ca^{2+}]_i$ produced by ACh and TG were abolished by BAPTA and those produced by Ca^{2+} ionophore A23187 were partially buffered. BAPTA inhibited in a dose-dependent manner H^+ secretion induced by cholinergic and gastrinergic stimulants in the presence of cimetidine. A23187 increased the AP ratio to values similar to those obtained with ACh or TG and was not inhibited by BAPTA. BAPTA partially inhibited (40%) the increase in AP ratio induced by forskolin and histamine inspite of the complete inhibition of the Ca^{2+} response. BAPTA did not inhibit the response to 8-BR-cAMP. BAPTA inhibition of forskolin stimulation was reversed by A23187 and the response was potentiated. These results indicate that ACh and TG response are completely dependent on an increase of $[Ca^{2+}]_i$. The response to cAMP-dependent agonists histamine and forskolin depend both on Ca^{2+} and cAMP. For forskolin stimulation the response may be the result of a potentiation between Ca^{2+} and cAMP.

Introduction

Cholinergic and gastrinergic stimulants appear to induce gastric acid secretion by a dual mechanism involving the action of released histamine and a receptor-mediated direct effect on the oxyntic or parietal cell [1–4]. The response seems to be the result of potentiation between these two parallel pathways. While the histamine limb is mediated by cAMP [5,6], the direct action of the secretagogues appears to involve the participation of Ca^{2+} [4,7–9]. This latter action is associated with a transient increase in free cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$ [8–13]. Furthermore, cAMP-dependent stimulants (histamine and forskolin) also appear to increase $[Ca^{2+}]_i$ [10,12]. The relationship between this increase of $[Ca^{2+}]_i$ and the physiological response has not been established. It is clear that to identify Ca^{2+} as a second messenger in the response to secretagogues in gastric acid secretion, the criteria of identity of action and collectibility would need to be satisfied. According to the first criterion the stimulus should increase the intracellular concentration of the putative messenger, in this case, Ca^{2+}. There is evidence showing that cholinergic and gastrinergic secretagogues induce an elevation of $[Ca^{2+}]_i$ linked to extracellular Ca^{2+} entry and/or release from intracellular stores [8–13]. On the other hand, an increase in $[Ca^{2+}]_i$ pro-

Abbreviations: BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; 8-BR-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; ACh, acetylcholine; TG, tetragastrin; AP ratio, aminopyrine accumulation ratio; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; DB-cAMP, *N*^{6},2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate.

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duced by the cAMP-dependent secretagogues has been reported in isolated gastric glands and parietal cells by some authors [9,10,12] but not by others [11]. The criterion of identity of action establishes that an elevation of the second messenger provokes the physiological response. An increase in $[Ca^{2+}]_i$ produced by Ca^{2+} ionophore should mimic the effect of secretagogue stimulation. A corollary to this criterion would be that an inhibition of the increase in $[Ca^{2+}]_i$ induced by the secretagogue should inhibit the response. The only evidence that satisfies this criterion is the stimulation by ionophore A23187 in amphibian mucosa [14–16]. However, this action has not been demonstrated in mammalian systems [17]. Furthermore, the relationship between the increase in $[Ca^{2+}]_i$ observed with secretagogue stimulation and the acid secretory response has not yet been established.

In this paper, we have studied both criteria with an emphasis on the identity of action. In this sense we have used the Ca^{2+}-ionophore A23187 to induce an artificial elevation in $[Ca^{2+}]_i$. Furthermore, the link between secretagogues, Ca^{2+} and acid secretion has been investigated using an intracellular Ca^{2+} chelator, BAPTA. A preliminary account of this work has already been presented [18].

Methods

Gastric glands

New Zealand White rabbits, weighing 1–2 kg were used for all experiments. Isolated gastric glands were prepared as described previously [19]. Briefly, the stomach was perfused under high pressure until completely exsanguinated, the mucosa stripped from the muscle coat and minced to small pieces. The fragments were digested with collagenase (1 mg/ml, Sigma Type I) in a medium containing (in mM): NaCl, 132.4; KCl, 10; Na_2HPO_4, 5; NaH_2PO_4, 1.2; $CaCl_2$, 1.0; $MgCl_2$, 0.8; DTT, 0.5; glucose, 2 mg/ml; bovine serum albumin, 10 mg/ml. Pyruvate (1 mM) was added to prevent formaldehyde toxicity when using the acetoxymethyl derivatives of Ca^{2+} buffers or indicators [20,21]. The initial pH was 7.4. Digestion proceeded at 37°C for 15–20 min in an oscillating water bath. The gland suspension obtained was filtered through nylon mesh and rinsed three times in incubation medium without collagenase at room temperature.

Measurement of acid secretion

Acid secretion by isolated gastric glands was measured by the [^{14}C]aminopyrine accumulation ratio ([^{14}C]AP) [19]. Glands were equilibrated in incubation medium containing 0.1 μCi/ml [^{14}C]AP during 45 min. Aliquots of this suspension were distributed in plastic falcon flasks and incubated at 37°C in a shaking water bath. Agents were added and after an appropriate incubation time, the suspension was briefly centrifuged in a microcentrifuge and the supernatant withdrawn. Pellets were dried overnight, weighed and dissolved in 200 μl of HNO_3 (20%) for 1 h at 75°C. Samples of dissolved tissue and supernatant were counted in a liquid scintillation spectrometer. [^{14}C]AP trapped in extracellular water was estimated from measurements of inulin space in aliquots of gland suspensions treated as for [^{14}C]AP measurements. Student's t-test was used for a statistical comparison of AP ratios between two means. ANOVA and Dunnet's tests were used for comparison of multiple treatments with a single control.

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

$[Ca^{2+}]_i$ was measured using the fluorescent calcium indicators, quin2 and fura-2, which were incorporated intracellularly as its acetoxymethyl esters, quin2/AM and fura-2/AM [20,22–24]. Glands (1–2 mg dry weight/ml) were incubated with 25–50 μM quin2/AM or 5–10 μM fura-2/AM in the medium described above, for 20 min at 37°C. Indicator-loaded glands were diluted 3-fold in incubation medium and allowed to stand at room temperature until use. Just prior to measurement, glands were briefly centrifuged and resuspended in the same medium, except for indicator and bovine serum albumin, which were omitted. Fluorescence was measured at 37°C in an Aminco Bowman spectrofluorometer equipped with a stirrer and polarizing filter. For quin2, the excitation and emission wavelengths were 335 and 495 nm, respectively. The intracellular free calcium concentration was calculated by the digitonin-EGTA method of Tsien et al. [23]. Quantification of $[Ca^{2+}]_i$ in glands when using quin2 was sometimes difficult due to fragmentation of glands by digitonin which introduced artifacts. Thus, these experiments in glands are reported as arbitrary fluorescence changes. Fura-2 fluorescence was used to measure transient changes in $[Ca^{2+}]_i$ induced by secretagogues which could not be observed with quin2 due to its buffering action. The excitation maxima for emission at 510 nm were 340 nm for the Ca^{2+}-bound and 355 nm for the Ca^{2+}-free indicator with a Ca^{2+}-insensitive excitation at 360 nm. The fluorescent intensities at 340 and 380 nm were measured by rapidly changing the monochromator settings by hand. The quantification of $[Ca^{2+}]_i$ was made using digitonin and EGTA to obtain fluorescence ratios of Ca^{2+}-bound and -free fura-2. $[Ca^{2+}]_i$ was determined using the 340/380 ratios according to the equation of Grynkiewicz et al. [24] assuming an apparent K_d of 224 nM.

The effect of BAPTA on the increase of $[Ca^{2+}]_i$ induced by secretagogues is presented as changes in fluorescence intensity, since we were unable to actually measure $[Ca^{2+}]_i$ due to interference of BAPTA with fura-2 fluorescence. The entry of BAPTA-AM into the cell increased the excited fluorescence at both 340 and

380 nm. However, qualitative changes in fluorescence at 340 nm give an indication of the effect of BAPTA on secretagogue-induced changes in $[Ca^{2+}]_i$.

Histamine, gastrin C-terminal tetrapeptide TG, ACh, DB-cAMP, 8-BR-cAMP, collagenase type I, A23187, quin2/AM, Hepes and DTT were obtained from Sigma Chemical. Forskolin and fura-2/AM were from Calbiochem, BAPTA-AM from Molecular Probes and [^{14}C]aminopyrine and [^{14}C]inulin from New England Nuclear. Reagent-grade salts were from E. Merck, Darmstadt, F.R.G. Cimetidine was the kind gift of Dr. Michael E. Parsons (Smith Kline and French. U.K.).

Results

Secretagogue effects on acid secretion and $[Ca^{2+}]_i$

Acid secretion, as measured by AP ratio, was increased by ACh, TG, histamine, forskolin and 8-BR-cAMP (Fig. 1). However, the magnitude of this response was dependent on the nature of the stimulus. cAMP-dependent secretagogues (histamine, forskolin and 8-BR-cAMP) were more potent stimulants than ACh and TG. The response to the latter secretagogues

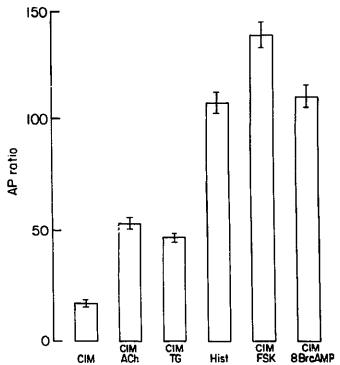

Fig. 1. Effect of ACh (10^{-3} M), TG (10^{-5} M), histamine Hist (10^{-4} M), forskolin (FSK, 5 μM) and 8-BR-cAMP (10^{-4} M), on the aminopyrine accumulation ratio (ΔAP ratio) in gastric glands. Glands were preincubated with [^{14}C]aminopyrine for 45 min before the addition of secretagogues. After 30 min of stimulation, the ΔAP ratio was measured as described in Methods. Cimetidine (CIM, 10^{-3} M) was present together with secretagogues except for histamine. Bars represent the mean ± S.E. of aminopyrine accumulation ratio in resting and secretagogue-stimulated glands in eight experiments (three replicates each). All stimulations were statistically significant in a paired t-test ($P < 0.001$).

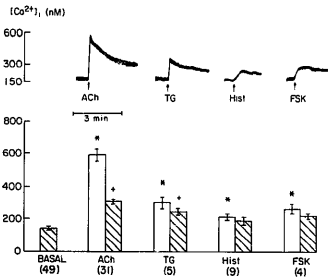

Fig. 2. Effect of ACh (10^{-3} M), TG (10^{-5} M), histamine (Hist, 10^{-4} M) and forskolin (FSK, 5 μM) on $[Ca^{2+}]_i$ in gastric glands. Glands were loaded with fura-2 and diluted as described in Methods. Traces on the upper part are representative recordings of changes in fura-2 fluorescence (340–510 nm, excitation-emission wavelengths) obtained from a single preparation. Ordinate is the $[Ca^{2+}]_i$ calculated from fluorescence ratios as described in Methods. Bars in the lower part represent the mean ± S.E. of $[Ca^{2+}]_i$ in resting and secretagogue-stimulated glands in n preparations (n in parenthesis). Open bars are peak values and hatched bars are sustained values after secretagogue stimulation. Basal value of $[Ca^{2+}]_i$ corresponds to the mean ± S.E. prestimulated $[Ca^{2+}]_i$ of all experiments. * Statistically significant difference in Student's paired t-test between peak and corresponding basal values ($P < 0.001$), + between peak and corresponding maintained values ($P < 0.01$).

represented about one-third of that obtained with cAMP-dependent stimulants.

As the experiments were performed in the presence of cimetidine to eliminate the endogenous histamine component, the response to ACh and TG should correspond to a direct effect of these secretagogues on the parietal cells and independent of cAMP.

Both cAMP-dependent and -independent secretagogues induced an increase in $[Ca^{2+}]_i$. However, the pattern of response varied according to the stimulant (Fig. 2). ACh and TG produced an elevation in $[Ca^{2+}]_i$ that was characterized by a rapid (< 3 s) and transient increase decaying to a sustained lower level. The amplitude of the transient response obtained with ACh was larger than that with any other secretagogue. Histamine and forskolin elicited an increase in $[Ca^{2+}]_i$, which was slower than with ACh and tetragastrin and was maintained, although there was a tendency for a slow decrease in the sustained level of Ca^{2+}. The cAMP analogue 8-BR-cAMP did not modify basal $[Ca^{2+}]_i$ (data not shown).

Acid secretion was more sensitive to cAMP-dependent than to cAMP-independent secretagogues. In contrast, ACh and TG increased $[Ca^{2+}]_i$ to higher levels than did histamine and forskolin. At first glance, it would seem from these experiments that there is no

correlation, or rather an inverse one, between the increase in $[Ca^{2+}]_i$ and acid secretion. However, we should take into account that cAMP is a potent stimulant of acid secretion and histamine and forskolin act via this pathway. At this point, we do not know how much of the secretory response is related to the increase in $[Ca^{2+}]_i$. On the other hand, ACh and TG provoked a similar rate of H^+ secretion with different increases in $[Ca^{2+}]_i$. This discrepancy may be due to the fact that we are observing changes in a mixed cell population which may have a differential sensitivity to secretagogues. Moreover, the role of the peak and sustained levels of Ca^{2+} in the secretory response has not been assessed. It has been proposed by Muallem et al. [13] that the sustained level of Ca^{2+} represents Ca^{2+} entry to the parietal cell and it is responsible for the stimulation of acid secretion. If this were the case, the similar levels of Ca^{2+} during the maintained phase of the response would explain the similar amplitude of acid secretion. However, it has been recently observed in purified preparations of peptic and parietal cells or in a single parietal cell of glands that both cell types show a biphasic increase in $[Ca^{2+}]_i$ with cholinergic and gastrinergic secretagogues. Nevertheless, we could consider that our results satisfy in a qualitative manner the criteria of collectibility, that is, the secretagogues increase $[Ca^{2+}]_i$. As the isolated gland preparation does not permit us to establish a correlation between $[Ca^{2+}]_i$ and acid secretion we have taken other approaches using a Ca^{2+} ionophore and intracellular chelators.

Ca^{2+}-ionophore effects on acid secretion

According to the criterion of identity of action, an elevation of $[Ca^{2+}]_i$ should mimic the effects of secretagogues. We have used the Ca^{2+}-ionophore A23187 to elevate $[Ca^{2+}]_i$. A23187 induced a dose-dependent increase in the AP accumulation ratio with a maximal effect at a concentration of 10 nM (Fig. 3). Ionophore concentrations higher than 20 nM gave submaximal responses until at 100 nM no effect was obtained. These experiments are in apparent contradiction with those of other authors who did not observe an acid secretory response with A23187 [13,17]. This discrepancy may be due to the high concentrations used by these authors. When high concentrations were used in our experiments (> 100 nM), no effect on acid secretion was observed. The stimulation of acid secretion by Ca^{2+}-ionophore A23187 supports the criterion of identity of action.

Effect of intracellular Ca^{2+} chelators on secretagogue-induced changes in $[Ca^{2+}]_i$ and on acid secretory response

In searching for a correlation between the increase in $[Ca^{2+}]_i$ and acid secretion during physiological stimulation with cAMP-dependent and -independent secretagogues, we have used intracellular Ca^{2+} chelators to

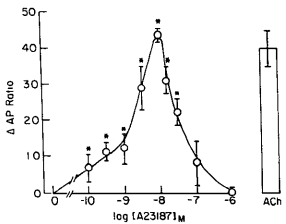

Fig. 3. Dose–response curve for A23187 on the aminopyrine accumulation ratio (ΔAP ratio). AP ratios were determined at all ionophore concentrations in each gland preparation in the presence of 10^{-3} M cimetidine 30 min after addition of A23187. Values are means ± S.E. for four experiments (three replicates each) for each point. Basal accumulation (cimetidine) was subtracted from each value for the elaboration of the graph. * Statistically different from basal (cimetidine) values in ANOVA + Dunnet's test ($P < 0.05$ at least). Bar represents the effect of ACh (10^{-3} M: mean ± S.E.) in the same experiments for comparison. The value for ACh was not significantly different from the value for 10 nM A23187 in a paired *t*-test.

buffer the changes in $[Ca^{2+}]_i$. We chose BAPTA as a buffer for its Ca^{2+} chelating properties in relation to others [22]. BAPTA was generated by intracellular esterases from BAPTA-AM added to the extracellular medium [20,23]. As BAPTA interfered with fura-2 and quin2 fluorescence, making the quantification of $[Ca^{2+}]_i$ unreliable in these conditions, we have evaluated the effect of buffers on $[Ca^{2+}]_i$ in resting or stimulated gastric glands using different concentrations of quin2 and fura-2. Increasing concentrations (5–50 μM) of fura-2/AM and quin2/AM generated an intracellular buffer concentration which did not modify $[Ca^{2+}]_i$ in the resting state after 30 and 60 min of incubation (results not shown). At shorter times (15 min) we observed a tendency to lower resting $[Ca^{2+}]_i$, especially at higher concentrations of quin2/AM. These experiments suggested that the intracellular buffer reduced transitorily the Ca^{2+} concentration. This effect was confirmed when BAPTA-AM was added to quin2-loaded cells (Fig. 4). BAPTA-AM induced a decrease in quin2 fluorescence followed by a slower return to the basal level. The amplitude of this decrease and its recovery was dependent on BAPTA-AM concentration. For 5 and 25 μM BAPTA-AM, the return to basal signal was achieved in a maximum of 15 and 45 min, respectively. For 50 μM recovery took 50–60 min. The second phase of increment of fluorescence toward basal levels suggested that the cell is able to regulate its $[Ca^{2+}]_i$ after perturbation. This would appear to be due to net calcium entry, since recovery of the basal fluorescence levels was not observed when Ca^{2+} was omitted from the

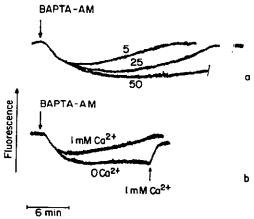

Fig. 4. Effect of BAPTA on quin2 fluorescence in gastric glands bathed in normal or Ca^{2+}-free solution. Glands were loaded with quin2 and diluted as described in Methods. After a stable basal fluorescence level was obtained, (a) BAPTA-AM (5–50 μM) was added at the arrow as indicated to glands suspended in normal Ca^{2+} medium. (b). 5 μM BAPTA-AM was added at the arrow in glands suspended in either normal or Ca^{2+}-free medium ($0Ca^{2+}$). In the latter case, 1 mM Ca^{2+} was added again to the cuvette after a few minutes. Values are representative of four different experiments.

extracellular solution but it was attained upon readdition of Ca^{2+} (Fig. 4b).

The action of BAPTA on secretagogue-induced changes in $[Ca^{2+}]_i$ was evaluated using fura-2. The effect of secretagogues in the presence of different concentrations of BAPTA-AM is presented as arbitrary fluorescence, since quantification is not possible in this condition (Fig. 5). BAPTA effectively buffered the increases in fluorescence induced by ACh, TG, histamine and forskolin. At 25 μM extracellular BAPTA-AM, intracellularly generated BAPTA was sufficient to eliminate the Ca^{2+} response induced by all secretagogues except for a small remnant with ACh. As BAPTA-AM is able to inhibit the increases in $[Ca^{2+}]_i$ in glands, this implies that, at least at high concentrations of buffer, the increases in $[Ca^{2+}]_i$ brought about by secretagogues in parietal cells must be inhibited.

Our experiments indicate that the intracellular Ca^{2+} chelators buffer the increase in $[Ca^{2+}]_i$ without modifying the resting level if enough time were allowed. Under these conditions, it should be possible to analyze the importance of the increase in $[Ca^{2+}]_i$ in the acid secretory response to different secretagogues.

Preincubation for 60 min with BAPTA-AM provoked an inhibition of the increase in AP ratio induced by ACh, TG, histamine and forskolin (Fig. 6). On the other hand, the Ca^{2+} chelator did not have a significant effect on the response to the cAMP analogue, 8-BR-cAMP. The inhibitory effect of BAPTA was stronger on the cAMP-independent secretagogues. While at 5 μM BAPTA-AM a significant reduction of the secretory response to ACh and TG was observed, 25 μM was necessary for a significant inhibition of forskolin and histamine stimulation. These effects can be best compared when percentage of stimulation is plotted as a function of BAPTA-AM concentration (Fig. 7). There were three groups of secretagogues according to their sensitivity to BAPTA-AM. One group (ACh and TG) was strongly inhibited by BAPTA. In this group, the effects of BAPTA on AP ratio were correlated with its

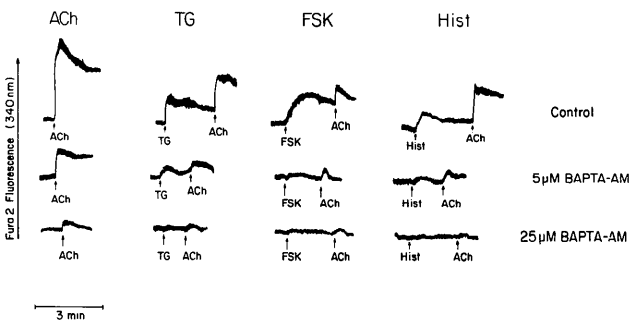

Fig. 5. Effect of BAPTA on increases in fura-2 fluorescence changes induced by secretagogues. Isolated gastric glands were loaded with fura-2 as described in Methods. After that, the glands were preincubated for 60 min at 37°C with different concentrations of BAPTA-AM (0.5, 25 μM). After washing of extracellular BAPTA-AM, fluorescence changes in response to ACh (10^{-3} M), TG (10^{-5} M), histamine (Hist, 10^{-4} M) and forskolin (FSK, 5 μM) were measured at an excitation of 340 nm (emission 510 nm). As intracellular BAPTA interfered with the calibration of the fura-2 signal, arbitrary fluorescence is shown. Recordings of changes in fura-2 fluorescence in glands from the same preparation. Values are representative of four experiments.

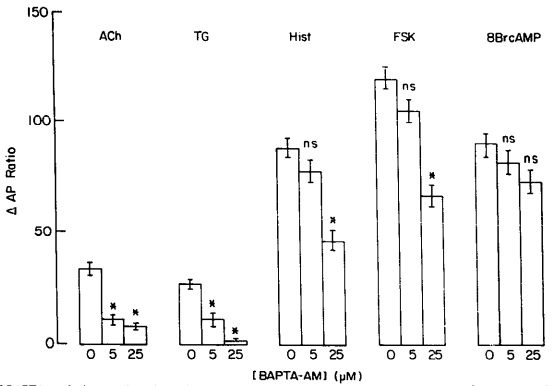

Fig. 6. Effect of BAPTA on the increase in aminopyrine accumulation ratio (ΔAP ratio) elicited by ACh (10^{-5} M), TG (10^{-5} M), histamine (Hist, 10^{-4} M), forskolin (FSK, 5 μM), 8-BR-cAMP (10^{-4} M). Isolated gastric glands were preincubated with [^{14}C]aminopyrine and BAPTA-AM (0, 5, 25 μM) during 60 min. Secretagogues were added in the continuous presence of BAPTA-AM for 30 min more. At the end of the stimulation period, the AP ratio was measured as described in Methods. Cimetidine (CIM, 10^{-3} M) was present together with secretagogues except for histamine. The ΔAP ratio was obtained by subtracting basal cimetidine value from each preparation from secretagogue-stimulated values. Bars correspond to the mean ± S.E. for four experiments (three replicates each). * Statistically significant difference with ANOVA + Dunnett's test between AP ratio in the presence or in the absence of BAPTA-AM, ($P < 0.05$ at least), n.s., non significant.

effect on intracellular Ca^{2+} change. At 25 μM, BAPTA-AM abolished acid secretion and Ca^{2+} increase induced by TG. At the same concentration of BAPTA-AM, ACh was able to induce a small response in both AP accumulation and $[Ca^{2+}]_i$. This suggests that the secretory response to ACh and TG is strongly dependent on the increase of $[Ca^{2+}]_i$ elicited by themselves. Regarding the cAMP-dependent secretagogues, histamine and forskolin, the inhibition of acid secretion by BAPTA was only partial in spite of a complete elimination of the Ca^{2+} response. The acid secretory response to these secretagogues was then partially dependent on an increase in $[Ca^{2+}]_i$. The fraction of acid secretion insensitive to BAPTA is most likely to be due to an increase in $[cAMP]_i$ brought about by these secretagogues. Furthermore, BAPTA did not have a significant effect on acid secretion induced by 8-BR-cAMP which did not produce any increases in $[Ca^{2+}]_i$. In this case, the acid

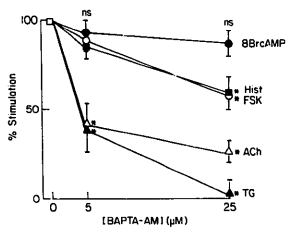

Fig. 7. Comparison of the effect of BAPTA-AM on the percentage of stimulation induced by the different secretagogues. Concentrations were the same as used in Fig. 6. Values are means ± S.E. of percentage of stimulation in the presence of different concentrations of BAPTA-AM relative to its own control value without BAPTA-AM. Data were calculated from individual experimental values presented in Fig. 6. * Significantly different from 100% values without BAPTA ($P < 0.05$ at least), n.s., non significant.

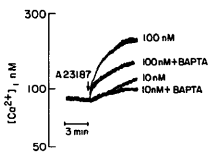

Fig. 8. Effect of A23187 on $[Ca^{2+}]_i$ in the presence or absence of BAPTA. Recordings of changes $[Ca^{2+}]_i$ in gastric cells were measured by quin2 fluorescence (see Methods) induced by 10 or 100 nM A23187 values are representative of three different preparations.

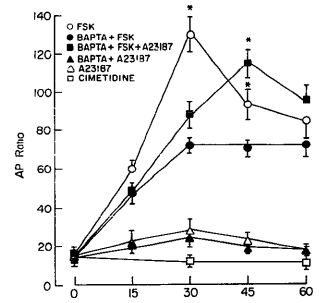

Fig. 9. Time-course of the effect of 5 μM forskolin and 10 nM A23187 in the presence or absence of 25 μM BAPTA-AM. All experiments were performed in the presence of 10^{-3} M cimetidine. Glands were preincubated with BAPTA-AM (25 μM) 60 min prior to the addition of stimulants. Values are means ± S.E. of four experiments (three replicates each). * Significantly different with respect to the group treated with BAPTA-AM and forskolin at each time-point ($P < 0.01$).

response would be dependent only on the increases in $[cAMP]_i$.

The fact that BAPTA-AM partially inhibits the amplitude of the AP ratio response to cAMP-dependent secretagogues indicated that the inhibited fraction is associated with the increase in $[Ca^{2+}]_i$. Since A23187 increases $[Ca^{2+}]_i$ even in the presence of BAPTA (Fig. 8), we have attempted to reverse the inhibitory effect of the chelator on forskolin stimulation using A23187. The time-course of forskolin stimulation in the presence or absence of BAPTA and the effect of Ca^{2+} ionophore on these conditions are presented in Fig. 9. Forskolin induced an increase in AP ratio which peaked at 30 min and maintained at a lower level. In the presence of 25 μM BAPTA-AM, forskolin induced a stimulation which was partially inhibited. The response was maintained at a level not different from that reached at 60 min without BAPTA-AM; the transitory fraction of the forskolin response was inhibited by the chelator. The response to forskolin and A23187 in the presence or absence of BAPTA-AM presented a transitory phase of an amplitude not significantly different from that observed with forskolin alone; however, the response was slower. The effect of ionophore alone in the presence of BAPTA-AM was small but significant. These results support the conclusion drawn earlier than an important fraction of the forskolin response is dependent on an increase in $[Ca^{2+}]_i$ elicited by cAMP-dependent stimulants.

Discussion

The aim of this work was to analyze the involvement of Ca^{2+} in the response to secretagogues according to the criteria of collectibility and identity of action. These criteria have been widely used to show the involvement of neurotransmitters in central nervous system functions [25]. In the course of this work, we have presented evidence that supports these criteria for the cAMP-independent secretagogues, ACh and tetragastrin, as well as the cAMP-dependent secretagogues, histamine and forskolin. These stimulants increased $[Ca^{2+}]_i$ and acid secretion in our preparation of isolated gastric glands.

Although AP accumulation is an index of parietal cell activity, the increase in $[Ca^{2+}]_i$ brought about by secretagogues should reflect the response of both peptic and parietal cells in the gland. However, part of the Ca^{2+} response to ACh, TG and forskolin observed by us should be taking place in the parietal cell; cholinergic and gastrinergic secretagogues and forskolin have been shown to increase $[Ca^{2+}]_i$ in purified parietal cell preparations [10] and in single parietal cells of isolated glands [12]. Moreover, the change in $[Ca^{2+}]_i$ induced by histamine must be occurring in the parietal cells, since this secretagogue specifically stimulates this cell type. Our results do not permit us to assess the source of Ca^{2+} for the increase in both cell types. However, it has been shown that in both peptic and parietal cells, the change in $[Ca^{2+}]_i$ involves the release of Ca^{2+} from intracellular stores and extracellular Ca^{2+} entry [10,12]. We have observed a reduction in the Ca^{2+} response to ACh after a first stimulation with TG, forskolin and histamine (Fig. 4), suggesting that these secretagogues may release Ca^{2+} from the same intracellular pool. This supports previous observations by other groups [9,10,12]. We can conclude that all secretagogues used here (except 8-BR-cAMP) increase $[Ca^{2+}]_i$ in the gland and most probably in the parietal cell. A quantitative relationship between acid secretion and $[Ca^{2+}]_i$ increase cannot be established in view of the existence of two main cell types in the gland preparation.

The use of Ca^{2+} ionophore and the intracellular Ca^{2+} chelator, BAPTA, has permitted us to analyze the relationship between Ca^{2+} and acid secretion in glands (criterion of identity of action). The stimulation of acid secretion by Ca^{2+} ionophore observed here is, in itself, evidence that Ca^{2+} can activate the secretory process. This activation has been shown previously in amphibian gastric mucosa [14–16] but not in mammalian preparations [13,17]. This negative observation may have been caused by the use of high concentrations of A23187 (1 μM), which upon acting as a protonophore would collapse the proton gradient developed across the apical membrane. We have observed stimulation of AP accumulation at low concentrations of A23187 (0.1–50 nM) but not at higher ones. Perhaps at low concentrations

the ionophore does not reach the organelles and apical membrane in a sufficient amount to cause a dissipation of the H^+ gradient. In this context we should mention that we have only observed exhaustion of intracellular Ca^{2+} pools at high concentrations of A23187 (1 μM) (unpublished data).

The inhibitory effect of BAPTA on stimulation induced by ACh, TG, histamine and forskolin represents strong evidence in favor of Ca^{2+} as a second messenger in the secretory response. The effect of BAPTA was that of buffering the Ca^{2+} transients induced by secretagogues. If enough time was allowed (45–60 min), the transitorily reduced resting levels returned to normal. The restoration of the basal concentration of Ca^{2+} was shown to be dependent on the entry of extracellular Ca^{2+}. This is what should be expected in a regulated system [23,26]. The resting $[Ca^{2+}]_i$ results from a balance between leaking and pumping rate. Addition of buffer decreases $[Ca^{2+}]_i$, thus activating the regulatory mechanisms which, in turn, trigger an increase in Ca^{2+} entry and/or a decrease in pump rate.

The inhibitory effects of BAPTA do not appear to be due to a toxic or side-effect of this compound. The formation of formaldehyde resulting from the hydrolysis of BAPTA-AM does not seem to be responsible for its effects. In control experiments, formaldehyde up to 2 mM did not inhibit the secretory response to 8-BR-cAMP. Moreover, BAPTA did not inhibit the secretory response to cAMP analogues (Fig. 8) nor basal O_2 consumption (data not shown). On the other hand, we have observed that the inhibition by BAPTA of forskolin stimulation is reversible upon addition of Ca^{2+}-ionophore A23187. Therefore, the effects of BAPTA-AM do not appear to be unspecific but are rather due to intracellular Ca^{2+} buffering by BAPTA.

The differential action of BAPTA on the secretory response permits the definition of three groups of secretagogues. In the first group belong ACh and TG where BAPTA inhibited both the Ca^{2+} and secretory responses. The small difference seen between secretagogues may be due to a larger ability of ACh to increase $[Ca^{2+}]_i$. These secretagogues do not increase cAMP concentration and appear to depend here solely on the increase in $[Ca^{2+}]_i$. It has been shown that ACh increases the production of inositol triphosphate (IP_3) [10,27]; the release of Ca^{2+} by IP_3 may be one of the mechanisms by which ACh increases $[Ca^{2+}]_i$. In the generation of IP_3 from phosphoinositides, diacylglycerol is also produced, which in turn activates protein kinase C (see Ref. 28 for a review). In many systems the physiological response appears to be the result of the interaction between the Ca^{2+}-activated and the protein kinase C-activated pathways [29]. In isolated pancreatic acini, carbachol induced a secretory response larger than that obtained with ionomycin despite similar increases in $[Ca^{2+}]_i$. This may be due to the interaction between Ca^{2+} and protein kinase C in the case of carbachol stimulation [30]. In our experiments, buffering of the ACh and TG-induced change in $[Ca^{2+}]_i$ led to an inhibition of the secretory response. However, at high BAPTA-AM concentration, the small secretory response to ACh may be related to a residual increase in $[Ca^{2+}]_i$, not completely buffered by BAPTA (Fig. 5). Also, this fraction of the response might be linked to protein kinase C activation by diacylglycerol. In this sense, it is worth mentioning that diacylglycerol is produced during carbachol stimulation of parietal cells and that protein kinase C activators can increase secretory activity under controlled conditions [27,31]. On the other hand, maximal responses to ACh, TG and A23187 are rather similar. Therefore, ACh and TG responses appear to be dependent primarily on the increase in $[Ca^{2+}]_i$. Thus, cholinergics and gastrin would be Ca^{2+}-dependent/cyclic AMP-independent secretagogues.

Histamine and forskolin also increase both $[Ca^{2+}]_i$ and acid secretion. The inhibition by BAPTA of the increase in $[Ca^{2+}]_i$ and partial inhibition of secretion indicate that Ca^{2+} participates significantly in the response to these secretagogues. On the other hand, the lack of effect of BAPTA on the secretion stimulated by 8-BR-cAMP and the large fraction of histamine and forskolin untouched by the Ca^{2+} chelator, confirms the importance of cAMP as a messenger in stimulation by these secretagogues. Taking into account the maximal secretion produced by calcium alone and the BAPTA-inhibited fraction (Ca^{2+}-dependent) of histamine- and forskolin-stimulated secretion, it would appear from simple calculations that potentiating interactions between Ca^{2+} and cAMP may account for the response. Clearly, a detailed analysis is required to define the quantitative participation of each messenger in the response. Thus, histamine and forskolin would conform to a second group of secretagogues being Ca^{2+}- and cAMP-dependent.

The response to agents that elevate cAMP bypassing the receptor and adenylate cyclase, 8-BR-cAMP and DB-cAMP, does not depend on an elevation of $[Ca^{2+}]_i$. They are thus Ca^{2+}-independent/cAMP-dependent.

The elevation of $[Ca^{2+}]_i$ by histamine and forskolin makes the mechanisms for this increase unclear. The lack of effect of 8-BR-cAMP would suggest production of cAMP is not necessary to trigger the increase in $[Ca^{2+}]_i$. The binding of histamine to the H_2 receptor could activate a G protein, different from the G_s, of the adenylate cyclase complex, which could be coupled to a phospholipase. Furthermore, forskolin not only activates the catalytic subunit of adenyl cyclase, but also the G_s proteins of the enzyme complex [32]. In this case it may also act on other G proteins regulating a phospholipase. Neither the finding nor these hypotheses can rule out at present the possibility of an increase in Ca^{2+} elicited by

cAMP. As pointed out by Negulescu and Machen [12], the increase in cAMP brought about by 8-BR-cAMP and DB-cAMP may be much slower than that produced by histamine and forskolin, dampening the effect that cAMP may have on intracellular Ca^{2+} levels.

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