Br. J. Pharmac. (1979), **67**, 123–131

INVESTIGATION OF THE ROLE OF EXTRACELLULAR CALCIUM IN THE CONTROL OF ACID SECRETION IN THE ISOLATED WHOLE STOMACH OF THE RAT

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1 An isolated stomach preparation from immature rats has been used to study the role of extracellular calcium in the control of gastric acid secretion. Calcium was removed from both the serosal and mucosal solutions either in the absence of a chelating agent or in the presence of EGTA.

2 Removal of calcium in the absence of EGTA had no significant effect on basal acid secretion or the acid responses to gastrin and dibutyryl cyclic adenosine 3',5'-monophosphate (db cyclic AMP). Under the same conditions there was a marked potentiation of the acid response to histamine, and a reduction of the acid response to acetylcholine which was readily reversed on restoring calcium to the bathing solutions.

3 Removal of calcium in the presence of EGTA caused an inhibition of basal acid secretion and of the acid responses to histamine and db cyclic AMP. In each case this reduction in acid output was readily reversed on bathing the stomachs in normal calcium-containing (2.5 mM Ca^{2+}) EGTA-free solutions.

4 The inhibition of the acid response to histamine produced by Ca^{2+}-free solutions which contained EGTA was not reversed on bathing the stomachs in solutions that contained both EGTA (0.1 mM) and an excess of calcium (2.5 mM).

5 The removal of extracellular calcium in the absence of EGTA provided evidence that the secretion of H^+ ions is dependent under some conditions on calcium ions. The possibility cannot be excluded that EGTA itself exerts an inhibitory influence on the process of acid secretion.

Introduction

A large volume of evidence now exists to indicate that calcium is an intracellular messenger in secretory cells, serving as a coupling factor between excitation and secretion. Calcium appears to be of particular importance in the control of the secretion of substances which are stored in intracellular vesicles or granules. Thus calcium is involved in the secretion of enzymes, polypeptide hormones, amines and acetylcholine (Case, 1973; Baker, 1974; Foreman, Garland & Mongar, 1976; Rasmussen & Goodman, 1977).

The role of calcium in electrolyte secretion is less clear. In the submandibular gland and pancreas of the cat, calcium depletion does reduce electrolyte secretion (Douglas & Poisner, 1963; Argent, Smith & Case, 1976), but Case (1973) has suggested that this is the result of an effect on membrane permeability and that calcium may not be involved in stimulus-secretion coupling. However there is some evidence that calcium is important in the control of electrolyte secretion from the salivary gland of the blow-fly and the rat parotid gland (evidence summarised by Berridge, 1975), and of cholecystokinin- or caerulein-induced electrolyte secretion from the perfused rat pancreas (Petersen & Ueda, 1976; Kanno & Yamamoto, 1977).

Extracellular calcium appears to be an important factor in the control of both basal and secretagogue-induced acid secretion in amphibian (Jacobson, Schwartz & Rehm, 1965; Kasbekar, 1974) and mammalian (Black & Welch, 1977; Main & Pearce, 1977a) gastric mucosa preparations. In the present study the role of extracellular calcium in the control of acid secretion has been studied in the rat isolated stomach preparation. A preliminary account of this work was given to the British Pharmacological Society (Bunce, Honey & Parsons, 1978).

Methods

Gastric acid secretion in the isolated stomach of the immature rat (35 to 45 g) was measured by the

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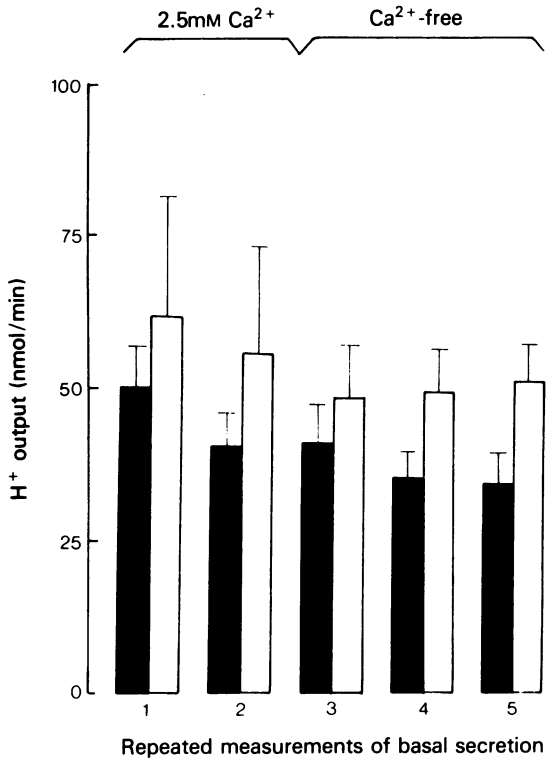

Figure 1 The effect of calcium-free solutions on basal acid secretion. The filled columns represent the control acid secretion (n = 6). The open columns represent the test procedure as indicated at the top of the figure (n = 6). Vertical lines show s.e. mean.

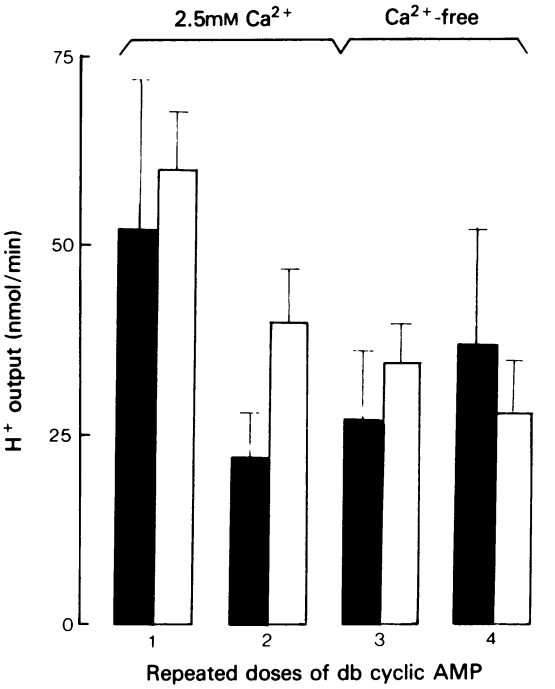

Figure 2 The effect of calcium-free solutions on the acid responses to repeated doses of 0.1 mM db cyclic AMP. The filled columns represent the control acid secretion (n = 7). The open columns represent the test procedure as indicated at the top of the figure (n = 6). Vertical lines show s.e. mean.

methods described by Bunce & Parsons (1976) and Bunce, Parsons & Rollings (1976). In brief, the rats were anaesthetized with pentobarbitone, the stomach exteriorised and the oesophagus ligated. An incision was made in the rumen of the stomach, and the contents washed out with warm Krebs-Henseleit solution. A second incision was made at the pyloric sphincter and polythene cannulae were inserted and tied into the stomach via these incisions. The stomach was rapidly dissected out and placed in Krebs-Henseleit solution at 37°C.

The lumen of the stomach was perfused at a rate of 1 ml/min with a modified Krebs-Henseleit solution from which the buffers ($NaHCO_3$ and KH_2PO_4) were omitted. The hydrogen ion concentration of the effluent perfusate from the stomach was continuously recorded, and the rate of acid secretion expressed as nmol per min. After setting up the stomach preparation the basal H^+ output was allowed to stabilize before the secretory responses to an agonist were investigated. The gastric secretagogues were added in a volume not exceeding 0.5 ml to the Krebs-Henseleit solution bathing the serosal surface of the stomach. The response to a single dose of an agonist was calculated as the amount of acid secreted at peak response minus the preceding basal level. On altering the calcium and/or EGTA content of the bathing solutions, the stomachs were equilibrated in these solutions for at least 1 h before further doses of the secretagogues were given.

Fresh solutions of histamine, dibutyryl cyclic adenosine 3′,5′-monophosphate (db cyclic AMP), acetylcholine and the calcium-chelating agent, ethyleneglycol-bis-(β-aminoethylether)N,N' tetraacetic acid (EGTA), were made up each day in Krebs-Henseleit solution. A stock solution of gastrin at 2.5 mg/ml was prepared in 0.05 M NH_4HCO_3. This solution was stored at −5°C and diluted with Krebs-Henseleit solution as required.

Drugs

The following drugs were used: pentobarbitone (Sagatal, May & Baker Ltd.), histamine acid phosphate and acetylcholine chloride (BDH Ltd.), db cyclic AMP

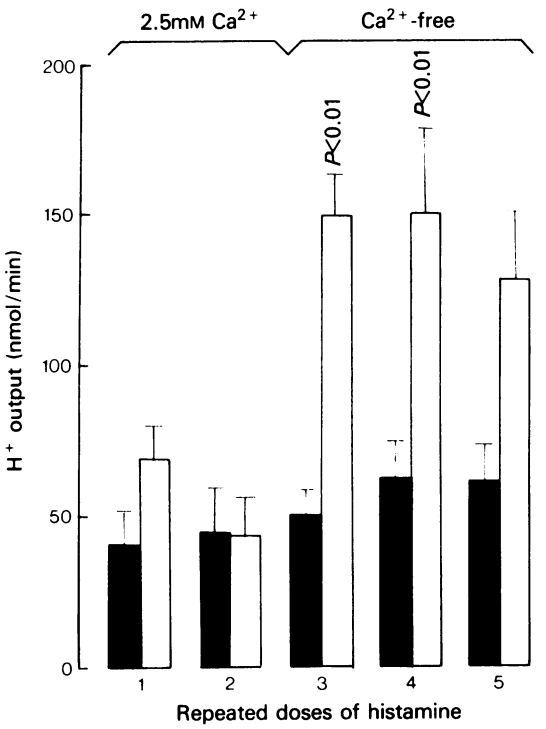

Figure 3 The effect of calcium-free solutions on the acid responses to repeated doses of 30 μM histamine. The filled columns represent the control acid secretion ($n = 6$). The open columns represent the test procedure as indicated at the top of the figure ($n = 6$). Vertical lines show s.e. mean.

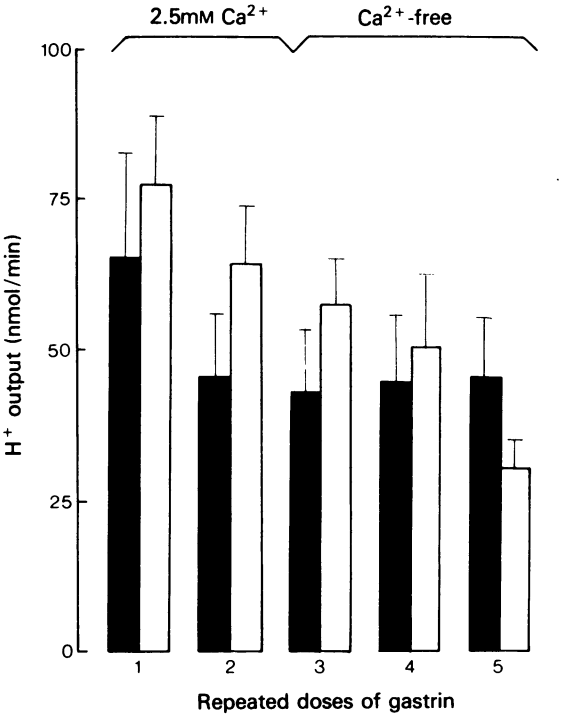

Figure 4 The effect of calcium-free solutions on the acid responses to repeated doses of 0.1 μM gastrin. The filled columns represent the control acid secretion ($n = 6$). The open columns represent the test procedure as indicated at the top of the figure ($n = 7$). Vertical lines show s.e. mean.

and EGTA (Sigma Chemical Co. Ltd.), gastrin (synthetic human gastrin I, Research Plus Laboratories Inc.).

Analysis of results

Results are expressed as mean ± s.e. mean. In some experiments the test procedure completely abolished acid secretion. Under these conditions the assumptions made for Student's *t*-test are not valid and therefore in the present work the difference between two samples was examined statistically by the Mann-Whitney U test as described by Siegel (1956). A two-tailed test was used. A P value of less than 0.05 was considered to be significant. When the difference between two samples is significant the P value is shown in the appropriate text figure.

Results

In the present experiments acid secretion was stimulated by giving repeated doses of the appropriate secretagogue, each dose being washed out when it had produced its peak secretory response and the acid secretion then allowed to return to a steady basal level before giving a further dose. Five doses of histamine (30 μM), gastrin (0.1 μM) and acetylcholine (1 mM) or four repeated doses of db cyclic AMP (0.1 mM) were administered. The basal acid secretion was always measured immediately before giving a dose of histamine. The calcium chelating agent, EGTA, was used at a concentration of 0.1 mM.

Control acid secretion

Control experiments were carried out in which the stomachs were incubated throughout the whole of the experimental period in solutions (serosal and mucosal) which contained 2.5 mM calcium. The output of acid under these conditions is recorded in all of the figures so that the acid secretion during a test procedure can be compared with the control.

The effect of calcium removal on acid secretion

The importance of extracellular calcium in the control of acid secretion was first investigated by simply

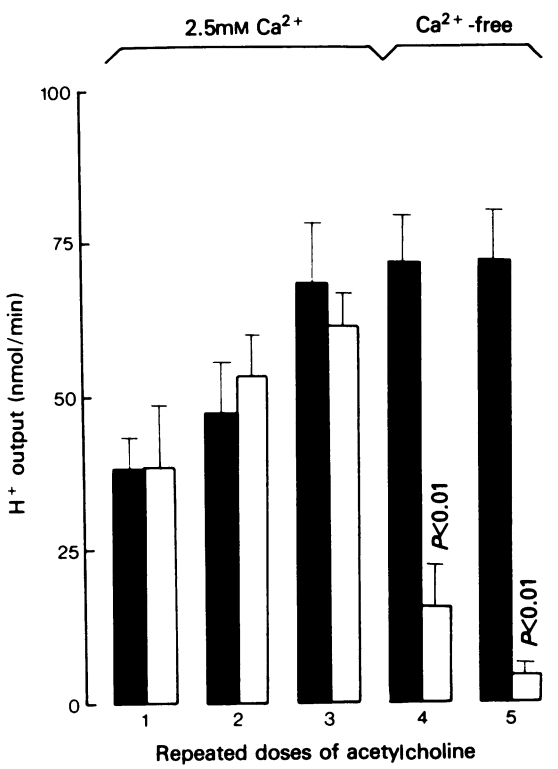

Figure 5 The effect of calcium-free solutions on the acid responses to repeated doses of 1 mM acetylcholine. The filled columns represent the control acid secretion (n = 6). The open columns represent the test procedure as indicated at the top of the figure (n = 8). Vertical lines show s.e. mean.

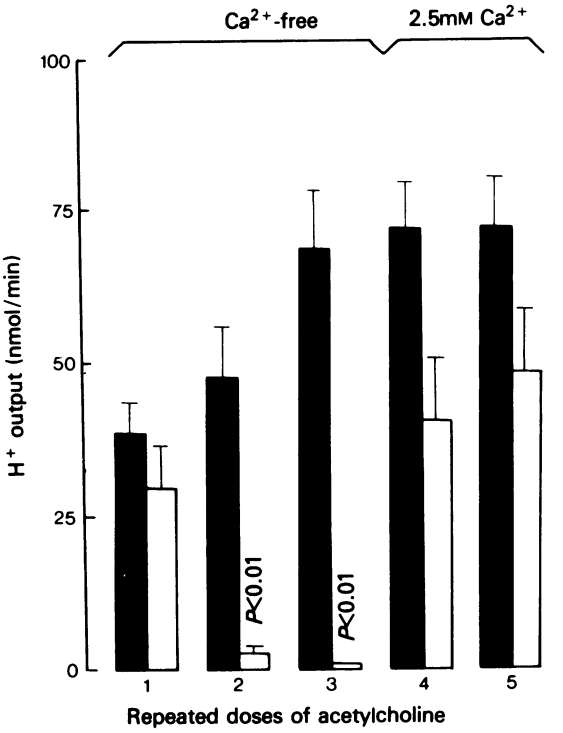

Figure 6 The effect of calcium (2.5 mM) on the acid responses to repeated doses of 1 mM acetylcholine in stomachs preincubated in calcium-free solutions. The filled columns represent the control acid secretion (n = 6). The open columns represent the test procedure as indicated at the top of the figure (n = 7). Vertical lines show s.e. mean.

removing calcium from both the serosal and mucosal solutions.

Basal secretion. In control experiments the basal acid secretion was measured five times during the mean experimental period of 4.44 h ($\pm$0.12, n = 6). In the test experiments two measurements of the basal secretion were made under control conditions followed by three measurements in the absence of calcium over a period of 5.60 h ($\pm$0.29, n = 6). The removal of calcium had no significant effect on the basal acid secretion (Figure 1).

Db cyclic AMP-stimulated secretion. In control experiments the four repeated doses of db cyclic AMP (0.1 mM) were given during the mean experimental period of 4.99 h ($\pm$0.16, n = 7). In test experiments two doses of db cyclic AMP were given under control conditions followed by two doses in the absence of calcium over a period of 5.10 h ($\pm$0.26, n = 6). Although there was some variability in the acid response to db cyclic AMP, the removal of calcium had no significant effect on the acid response to this secretagogue (Figure 2).

Histamine-stimulated secretion. In control experiments the five repeated doses of histamine (30 μM) were given during the mean experimental period of 4.44 h ($\pm$0.12, n = 6). In test experiments two doses of histamine were given under control conditions followed by three doses in the absence of calcium over a period of 5.60 h ($\pm$0.29, n = 6). The removal of calcium produced a significant increase in the acid response to histamine (Figure 3).

Gastrin-stimulated secretion. In control experiments the five repeated doses of gastrin (0.1 μM) were given during the mean experimental period of 3.57 h ($\pm$0.18, n = 6). In test experiments two doses of gastrin were given under control conditions, followed by three doses in the absence of calcium over a period of 4.44 h ($\pm$0.26, n = 7). In both the control and test

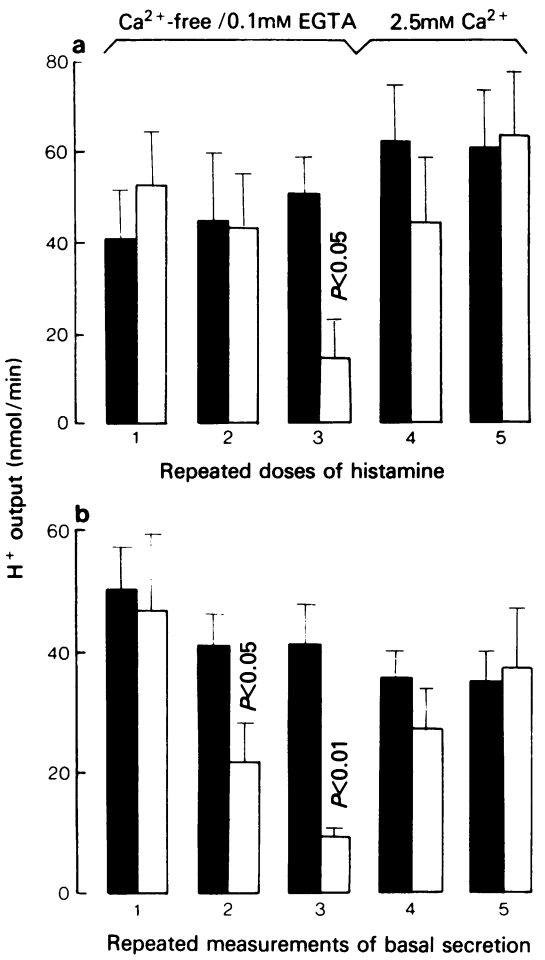

Figure 7 The effect on histamine-evoked acid secretion of calcium-free solutions containing 0.1 mM EGTA, and the effect of subsequent calcium (2.5 mM) in the absence of EGTA. The filled columns represent the control acid secretion. The open columns represent the test procedure as indicated at the top of the figure. Vertical lines show s.e. mean. (a) Acid response to repeated doses of 30 μM histamine (control, $n = 6$; test, $n = 7$); (b) basal acid secretion (control, $n = 6$; test, $n = 7$).

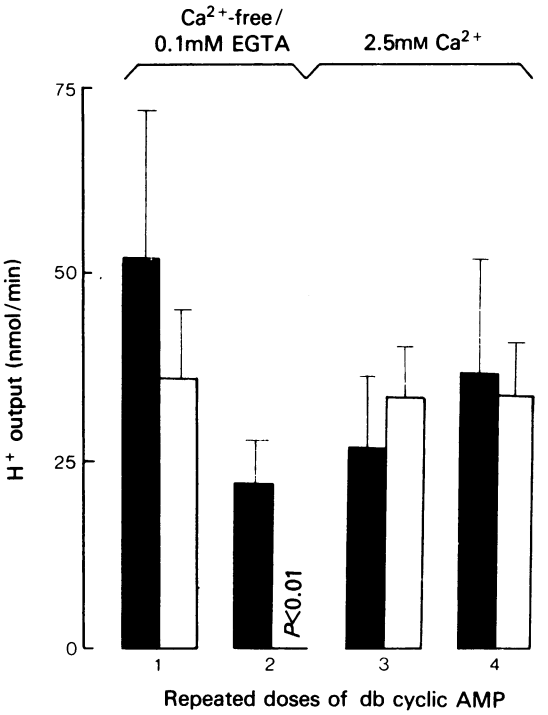

Figure 8 The effect of calcium-free solutions containing 0.1 mM EGTA on the acid responses to repeated doses of 0.1 mM db cyclic AMP, and the effect of the subsequent addition of calcium (2.5 mM) in the absence of EGTA. The filled columns represent the control acid secretion ($n = 7$). The open columns represent the test procedure as indicated at the top of the figure ($n = 6$). Vertical lines show s.e. mean.

experiments there was a tendency for the acid responses to gastrin to diminish during this time. Although this change was greater under test conditions there was no evidence to suggest that the removal of extracellular calcium had any significant effect on gastrin-stimulated secretion (Figure 4).

Acetylcholine-stimulated secretion. Initially, the effect of calcium depletion on the acid response to acetylcholine was investigated with an experimental design similar to that applied to the other secretagogues. In the control experiments the five repeated doses of acetylcholine (1 mM) were given during the mean experimental period of 3.24 h (±0.10, $n = 6$). In the test experiments three doses of acetylcholine were given under control conditions followed by two doses in the absence of calcium over a period of 3.77 h (±0.16, $n = 8$). The removal of calcium produced a significant inhibition of the acid response to acetylcholine (Figure 5).

The depletion of extracellular calcium can damage the structural integrity of the gastric mucosa and this possibility was investigated in the next group of experiments. In these experiments three doses of acetylcholine were given in calcium-free conditions followed by two doses in the presence of 2.5 mM calcium over a period of 3.77 h (±0.16, $n = 7$). The addition of calcium to the extracellular bathing fluid readily reversed the inhibition of the acid response to acetylcholine (Figure 6). This result shows that any tissue

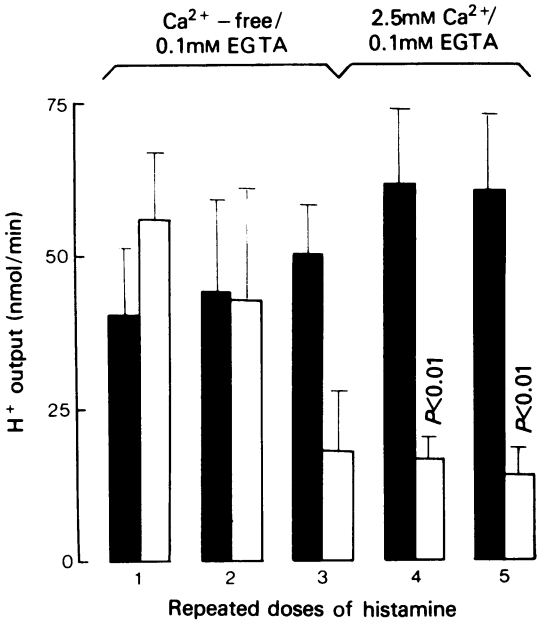

Figure 9 The effect of calcium-free solutions containing 0.1 mM EGTA on the acid responses to repeated doses of 30 μM histamine, and the effect of the subsequent addition of calcium (2.5 mM) in the presence of 0.1 mM EGTA. The filled columns represent the control acid secretion ($n = 6$). The open columns represent the test procedure as indicated at the top of the figure ($n = 6$). Vertical lines show s.e. mean.

damage that may have occurred was not of an irreversible nature.

The effect on acid secretion of calcium removal in the presence of EGTA

It is probable that the removal of calcium from the bathing solutions does not totally deplete extracellular calcium. A more rigorous procedure is required for the removal of the calcium present in the intercellular spaces or bound to the outside of the cell membrane (Schramm, 1976). For this reason the effect of calcium depletion in the presence of the chelating agent EGTA (0.1 mM) on basal acid secretion and the acid responses to histamine and db cyclic AMP was determined. Both the serosal and mucosal solutions were treated in this way. Basal acid output and histamine-stimulated secretion were studied in the same experiments; three doses of histamine (30 μM) were given in the absence of calcium (plus 0.1 mM EGTA) followed by two doses of histamine under normal conditions (2.5 mM calcium, no EGTA). The effect of calcium removal in the presence of EGTA on the acid response to db cyclic AMP was investigated in a separate group of experiments. Two doses of db cyclic AMP (0.1 mM) were given in calcium-free conditions followed by two doses in normal conditions (2.5 mM calcium, no EGTA). The results are shown in Figures 7 and 8. In calcium-free conditions histamine-stimulated acid secretion diminished from 52.6 to 15.0 nmol/min and basal acid output was reduced from 46.6 to 9.1 nmol/min over a period of 1.82 h (±0.13, $n = 7$) (Figure 7). The acid response to db cyclic AMP was more rapidly affected by EGTA than that to histamine, and acid secretion was diminished from 35.9 to 0.1 nmol/min over a period of 1.70 h (±0.14, $n = 6$) (Figure 8). On bathing the stomachs in normal calcium-containing solutions the basal acid secretion and the acid responses to both histamine and db cyclic AMP returned to control levels (Figures 7 and 8).

If the previous experiments with EGTA are to be clearly interpreted it is necessary to determine whether EGTA has any effect which is unrelated to its calcium-chelating properties. For this purpose experiments were carried out to investigate the effect of solutions (both serosal and mucosal) which contained both EGTA (0.1 mM) and an excess of calcium (2.5 mM) on histamine-stimulated acid secretion. In these studies (Figure 9) three doses of histamine in the absence of calcium (plus 0.1 mM EGTA) were given followed by two doses in the presence of both 2.5 mM calcium and 0.1 mM EGTA. A comparison of these data (Figure 9) with the results shown in Figure 7 shows that EGTA inhibits acid secretion even in the presence of a large excess of calcium; an observation which suggests that EGTA was producing an effect unrelated to its calcium-chelating properties.

Discussion

The present study has revealed some differences in the dependency of the various gastric secretagogues on extracellular calcium. The removal of calcium had no significant effect on the acid response to db cyclic AMP, and this result agrees well with the work of Main & Pearce (1978) on the rat isolated gastric mucosa preparation. Taken together, these results suggest that there is no requirement for extracellular calcium in the control of the acid response to db cyclic AMP, although this does not preclude the possibility that db cyclic AMP causes a mobilisation of intracellular calcium stores; an effect which is thought to occur in a number of secretory systems (Berridge, 1975).

Calcium depletion caused a potentiation of the acid response to histamine, and again this result agrees well with the work of Main & Pearce (1977a) using the rat isolated gastric mucosa, although Black &

Welch (1977) have found that removal of calcium had no significant effect on histamine-stimulated acid secretion in the isolated stomach of the mouse.

The result obtained in the present study using histamine is difficult to explain. It has been reported that in some systems adenylate cyclase activity is inhibited by relatively high concentrations of calcium (Steer, Atlas & Levitzki, 1975; Wiemer, Kaiser & Palm, 1978), and there is some evidence that histamine stimulates acid secretion via cyclic AMP (Scholes, Cooper, Jones, Major, Walters & Wilde, 1976; Soll & Wollin, 1977). It is possible that 2.5 mM is not the optimum concentration of calcium for the histamine-sensitive adenylate cyclase in rat gastric mucosa, and therefore under these conditions histamine does not produce its full response.

Calcium removal had no significant effect on the acid response to gastrin, and this result is in conflict with previous results obtained with mammalian stomach preparations. Main & Pearce (1977a) have reported that removal of calcium potentiates the acid response to pentagastrin in the rat isolated gastric mucosa preparation, and Black & Welch (1977) found that under similar experimental conditions using the mouse isolated stomach the acid response to pentagastrin was diminished. Kaplan & Peskin (1969) have reported that in the isolated gastric mucosa of the frog removal of calcium causes an inhibition of the acid response to pentagastrin.

There is a large volume of evidence to show that in the rat, gastrin (or pentagastrin) stimulates acid secretion via the mobilization of gastric mucosal histamine (evidence summarised by Henman, 1975). In this situation it might be expected that the removal of calcium would have the same effect on the response to gastrin as that to exogenous histamine, which of course was not observed. On the other hand the anaphylactic release of histamine from peritoneal mast cells is calcium-dependent (Foreman *et al.* 1976). If an analogy between anaphylaxis and gastrin-stimulated secretion can be made, it could be predicted that the removal of extracellular calcium would diminish the acid response to gastrin by impairing the release of endogenous histamine. Thus, although the inhibition of gastrin-stimulated acid secretion by the H_2-receptor antagonist metiamide suggests a histamine link (Bunce *et al.*, 1976) the present work does not contribute further to our understanding of the mechanism of action of gastrin. Of course, the possibility remains that histamine and gastrin stimulate acid secretion through completely separate pathways as suggested by Soll (1978), and that metiamide perhaps inhibits the acid response to gastrin through a type of receptor interaction which is allosteric in nature (Grossman & Konturek, 1974).

The observation that the acid response to acetylcholine is dependent on extracellular calcium contrasts with the results of Main & Pearce (1977b) using methacholine in the rat isolated gastric mucosa preparation. It has been reported that the cholinomimetic drug, carbamylcholine, stimulates oxygen uptake in isolated parietal cells (Soll, 1978) which suggests that acetylcholine may have a direct effect on the parietal cell. In the light of this direct action it could be argued from our own results that the interaction of acetylcholine with the cholinoceptors on the parietal cell causes an influx of extracellular calcium into the cell which mediates the secretion of H^+ ions. A study of $^{45}Ca^{2+}$ exchange in an isolated parietal cell preparation may resolve this question. The present results with acetylcholine are consistent with the *in vitro* pharmacological studies with H_2-receptor antagonists which indicate that the secretagogue action of acetylcholine is not mediated by histamine (Tepperman, Schofield & Tepperman, 1975; Bunce *et al.*, 1976; Wan, 1977).

Previous studies on the amphibian isolated gastric mucosa have shown that removal of calcium can cause structural damage to the secretory tissue (Sedar & Forte, 1964). However, the failure of calcium removal to diminish the acid responses to the other secretagogues indicates that the inhibition of the acid response to acetylcholine cannot be explained in this way.

Removal of calcium had no significant effect on the basal acid secretion. In contrast, Main & Pearce (1977a) have reported that calcium removal tends to increase the rate of basal acid output from the rat isolated gastric mucosa preparation, although the depletion of extracellular calcium causes an inhibition of the basal acid secretion in amphibian isolated gastric mucosa (Jacobson *et al.*, 1965). In the present work a comparison of the effects of calcium removal on basal secretion and on the acid responses to histamine and acetylcholine suggests that the basal secretion is under neither histaminergic nor cholinergic control. This interpretation of the data is in accordance with the observations that the basal secretion is resistant to inhibition by both metiamide and atropine (Bunce & Parsons, 1976; Bunce, Marsh & Parsons, 1977).

The studies with EGTA were designed to ensure the total removal of extracellular calcium, although in practice the results are difficult to interpret. The failure of an excess of calcium to reverse the inhibition seen in the presence of EGTA indicates that this inhibition is not the consequence of the depletion of extracellular calcium. Therefore it is possible that EGTA itself is having a direct effect on the gastric mucosa, and there are two possible mechanisms through which this may occur. Firstly EGTA may be altering the secretory properties of the gastric mucosa, although it must be pointed out that there is no evidence that EGTA has a general non-specific

effect on secretory systems (cf. studies in the pancreas; Argent, Case & Scratcherd, 1973). Secondly, the inhibition of acid secretion caused by EGTA may be the result of damage to the mucosal tissue. Indeed, Sedar & Forte (1964) have reported that the amphibian gastric mucosa is damaged in the presence of the chelating agent EDTA and that, as in our own experiments, this was readily reversed on the removal of the chelating agent and the readdition of calcium.

In summary, the removal of extracellular calcium in the absence of EGTA showed that the process of acid secretion is dependent, under some conditions, on calcium ions although the studies with EGTA did not contribute further to our understanding of the nature of this dependency.

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(*Received December 8, 1978.*)