An ATP-dependent and inositol trisphosphate-sensitive Ca^{2+} pool linked with microfilaments of the parietal cell

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In digitonin-permeabilized parietal cells, *myo*-inositol 1,4,5-trisphosphate (Ins P_3) or Ca^{2+} ionophore (A23187) increased the cytosolic Ca^{2+} concentration due to the intracellular Ca^{2+} release. Addition of ATP decreased the cytosolic Ca^{2+} concentration due to the rapid Ca^{2+} re-uptake into the same or similar pool which releases Ca^{2+} from a non-mitochondrial location (measured by quin2/AM and $^{45}Ca^{2+}$). Cytochalasin B failed to increase the cytosolic Ca^{2+} concentration in response to Ins P_3 or A23187 and even failed to decrease the cytosolic Ca^{2+} concentration in response to ATP. This implies that the ATP-dependent and Ins P_3-sensitive Ca^{2+} pool is linked with the microfilaments of the parietal cell. In intact parietal cells, A23187 increased the amino[^{14}C]pyrine accumulation (an index of acid secretion), that was independent of medium Ca^{2+}. This increase of acid secretion was inhibited by the pretreatment with cytochalasin B. This suggests that medium Ca^{2+}-independent acid secretion (by A23187) is regulated by the microfilaments. Therefore, there is a close relationship between the intracellular Ca^{2+} metabolism, microfilaments and acid secretion

myo-*Inositol 1,4,5-trisphosphate* *A23187* *ATP* *Microfilament* *Ca^{2+} pool* *Parietal cell (guinea pig)*

1. INTRODUCTION

Acid secretion from parietal cells is regulated by either cyclic AMP produced by a histaminergic pathway [1] or by increase in cytosolic Ca^{2+} concentration elicited by a cholinergic pathway [2–4] and possibly by stimulation with gastrin [5,6]. The response to cholinergic stimulation requires medium Ca^{2+} and causes Ca^{2+} entry from the extracellular space [2–4]. The intracellular Ca^{2+}-mobilizing hormone is thought to be gastrin since it induces an increase of aminopyrine accumulation or oxygen consumption (as an index of acid secretion) and an increase in cytosolic Ca^{2+} concentration, that are independent of medium Ca^{2+} [5,6]. This medium Ca^{2+}-independent Ca^{2+} release and acid secretion (by gastrin) was inhibited by cytochalasin B [6]. Cytochalasin B has been shown to cause a disruption of microfilaments consisting of an actin-like protein linked with meromyosin [7,8]. This indicates the possibility that gastrin-elicited Ca^{2+} release and acid secretion may be regulated by the function of microfilaments of the parietal cell. On the other hand, the increase in cytosolic Ca^{2+} concentration brought about by Ca^{2+}-mobilizing hormones such as adrenaline in hepatocyte, cholecystokinin in pancreatic acini, and others is mainly due to the release of Ca^{2+} from intracellular stores (endoplasmic reticulum) [9–13]. In these cells, Ins P_3 could be the second messenger linking the Ca^{2+}-mobilizing receptor activation to intracellular Ca^{2+} release [14–19]. This study has been

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Abbreviations: quin2/AM, 2-[2-amino-5-methylphenoxyl] methyl-6-methoxy-8-aminoquinoline-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; Ins P_3, *myo*-inositol 1,4,5-trisphosphate

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focused on defining the Ca^{2+} metabolism of the parietal cell, which may be regulated by Ins P_3 and ATP, and linked with microfilaments.

2. MATERIALS AND METHODS

2.1. *Materials*

The sources of some of the below-mentioned reagents have been given previously [5,6]. Ins P_3, antimycin, creatine phosphate, creatine phosphokinase, 2,4-dinitrophenol and digitonin were obtained from Sigma (USA).

2.2. *Cell separation*

Parietal cells were prepared by a modification of the method described in [20,21]. Gastric mucosae from a non-fasted male guinea pig of 300 g body wt (Hartley) were digested with 300 U collagenase I and 4800 U dispase II in 20 ml medium 199 (tissue culture medium which had been modified by Earle's medium) containing 2000 U penicillin-streptomycin mixture and 10% heat-inactivated fetal calf serum. The tissue was gassed continuously with 5% CO_2 (pH 7.4) and shaken for 60 min at 120 oscillations per min in a water bath at 37°C. The gastric glands obtained (by enzymatic digestion) were then mechanically disrupted by sequential passage through silicone-treated glass pipettes in 30 ml Krebs-Ringer bicarbonate buffer solution containing 0.2% glucose, 0.2% bovine serum albumin, 0.5 mM dithiothreitol and 1 mM EDTA but without Ca^{2+} and Mg^{2+} to obtain heterogenously dispersed mucosal cells. Further cell separation was achieved by the Beckman elutriation system (Beckman, USA) [6,21] and a parietal cell enriched fraction (proportion, 85%) was obtained with a flow rate of 48 ml/min and a centrifugal speed of 1450 rpm based on the Stokes-Einstein relation. The viability of parietal cells determined by exclusion of 0.4% Trypan blue was 95%.

2.3. *Cytosolic Ca^{2+} concentration in permeable cells*

Cytosolic Ca^{2+} concentration was measured by a described method [22]. Isolated parietal cells were immediately suspended at a density of 10^6 cells/ml in a medium resembling the cytosol which had the following composition (mM): NaCl, 40.6; KCl, 108; $MgSO_4$, 4.8; KH_2PO_4, 1.2; $NaHCO_3$, 25; EGTA, 1; $CaCl_2$, 0.49 in 15 mM Hepes (pH 7.2). The concentration of medium Ca^{2+} was set at 180 nM which is almost equivalent to the resting level of cytosolic Ca^{2+} concentration of the parietal cell (130–170 nM) [4,6]. The medium also contained 2% bovine serum albumin, 0.2% glucose and 20 μg/ml of digitonin. The cells were incubated for 10 min at 37°C in an atmosphere of 95% O_2/5% CO_2 (pH 7.2) and then centrifuged at $100 \times g$ for 5 min. The cells (10^6) were resuspended in 1 ml of the same medium without digitonin but with antimycin (10 μM), 2,4-dinitrophenol (10 μM) and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (50 μg/ml). This incubation medium was designated as 'cytosol buffer'. Quin2/AM (20 μM) was then added to the permeable parietal cells (10^6 cells/ml) in the cytosol buffer as described [5,6]. At the appropriate time, either ATP (1.5 mM), Ins P_3 (5 μM) or A23187 (5 μg/ml) was added to the incubation medium (10^6 cells/2 ml cytosol buffer) in a cuvette with stirring at 37°C, in a Hitachi 650-10L1 spectrometer (Hitachi, Japan). The excitation and emission wavelengths were 339 nm (4 band width) and 492 nm (10 band width), respectively. Cytosolic Ca^{2+} concentration was calculated from the formula; $[Ca^{2+}]_i = 115\ nM(F-F_{min})/(F_{max}-F)$. Cells were lysed by sonication and the fluorescence at high Ca^{2+} concentration (2.6 mM, F_{max}) and without Ca^{2+} (2 mM EGTA, F_{min}) was determined. F is the relative fluorescence measurement of the sample [22]. A23187 gave rise to its own fluorescence and the reading was subtracted from the experimental value.

2.4. *$^{45}Ca^{2+}$ fluxes in permeable cells*

Digitonin-permeabilized cells (10^6/ml, cytosol buffer) were loaded in 1.5 mM ATP to accumulate $^{45}Ca^{2+}$ (1.0 μCi, NEN, USA) at 37°C. Then, either Ins P_3 (5 μM) or A23187 (5 μg/ml) was added to the incubation medium at 20 min. The final reaction was stopped by adding 2 ml ice-cold cytosol buffer without isotope. The cell suspension was immediately placed on Millipore (pore size, 1.2 μm, RAWP) under mild suction (4.9 inches Hg) as described [5,6]. The cell suspension on the filter pads was washed 4 times with the cytosol buffer without isotope and the dried filter pads were digested by 200 μl distilled water and 500 μl Pro-

tosol (NEN) for 12 h. After adding 15 ml Aquasol (NEN), samples were counted for radioactivity in a liquid scintillation counter (Aloka, USA).

2.5. *Acid secretion by intact parietal cells*

The accumulation of amino[^{14}C]pyrine as an index of acid secretion in vitro was measured by the method of Berglindh [23]. Parietal cells (10^6) were incubated with the indicated agents plus 0.1 μCi amino[^{14}C]pyrine in 1 ml Krebs-Ringer bicarbonate buffer with or without medium Ca^{2+} for 10 min at 37°C. The Ca^{2+}-free medium was prepared by omitting $CaCl_2$ and $MgCl_2$, and by adding 1.0 mM EDTA in Krebs-Ringer bicarbonate buffer. The amino[^{14}C]pyrine trapped in the cells was determined by Millipore (pore size, 1.2 μm, RAWP) under mild suction as described [5,6]. The radioactivity remaining on the filter was counted as mentioned above. The value is expressed as the ratio of amino[^{14}C]pyrine trapped in the cell (see table 1, legend), [23].

2.6. *Treatment of cells with cytochalasin B*

A cell suspension (10^6/ml) consisting of either permeable cells or intact cells, which were suspended in cytosol buffer and Krebs-Ringer bicarbonate buffer, respectively, was incubated with cytochalasin B (10 μg/ml) for 20 min at 37°C prior to the measurements for Ca^{2+} fluxes (by quin2/AM or $^{45}Ca^{2+}$) and acid secretion (by amino[^{14}C]-pyrine).

3. RESULTS

The mechanism of intracellular Ca^{2+} metabolism in the parietal cell was studied after the permeabilization of cells by digitonin. As shown in fig.1, the cytosolic Ca^{2+} concentration at a resting level in digitonin-permeabilized cells was 152.8 ± 4.7 nM (n = 9), (measured by quin2/AM), which was not significantly different from that in intact parietal cells (172.4 ± 4.7 nM, n = 9, without medium Ca^{2+}; 202.6 ± 5.0 nM, n = 9, with medium Ca^{2+}, 1.3 mM) [5,6]. The first injection of ATP (1.5 mM) caused a substantial sequestration of Ca^{2+} into the pool, resulting in a decrease of cytosolic Ca^{2+} concentration (from 152.8 ± 4.7 to 131.8 ± 8.0 nM, n = 9). Following the addition of either Ins P_3 (5 μM) or A23187 (5 μg/ml), ATP-dependent Ca^{2+} uptake caused a

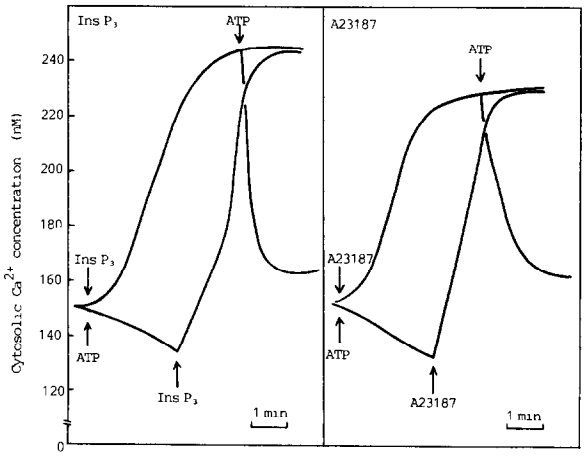

Fig.1. Ca^{2+} released by either Ins P_3 or A23187 and Ca^{2+} taken up (or re-taken up) by ATP in digitonin-permeabilized parietal cells as measured by quin2/AM. The concentrations of reagents tested were as follows; ATP, 1.5 mM; Ins P_3, 5 μM; A23187, 5 μg/ml. The data represent means of 9 determinations from 3 experiments.

rapid increase of cytosolic Ca^{2+} concentration due to Ca^{2+} release from the pool (Ins P_3, from 131.8 ± 8.0 to 248.2 ± 41.9 nM, n = 9; A23187, from 131.8 ± 8.0 to 226.1 ± 22.4 nM, n = 9). Cytosolic Ca^{2+} released by either Ins P_3 or A23187 was immediately taken up again into the pool when ATP was added (Ins P_3, from 248.6 ± 46.2 to 167.5 ± 24.1 nM, n = 9; A23187, from 226.9 ± 24.6 to 163.4 ± 16.7 nM, n = 9). The amount of Ca^{2+} removed by ATP when Ca^{2+} was released by either Ins P_3 or A23187 exceeded that by ATP when cytosolic Ca^{2+} concentration was maintained at the resting level (the amount of Ca^{2+} removed by ATP: the former, 81.2 nM for Ins P_3, 63.5 nM for A23187; the latter, 21.0 nM). These Ca^{2+} movements induced by Ins P_3, A23187 or ATP were insensitive to 2,4-dinitrophenol, which is known to uncouple the mitochondrial Ca^{2+} metabolism [17]. Cytochalasin B, which is known to disrupt the microfilaments consisting of actin-like protein [24,25] inhibited the Ca^{2+} release induced by Ins P_3 (reduced by 56.9%) and A23187 (reduced by 72.2%) and the concomitant Ca^{2+} uptake induced by ATP (fig.2). Especially, removal of Ca^{2+} by ATP was completely inhibited by cytochalasin B. Fig.3 shows the effects of Ins P_3 or A23187 on ATP-dependent $^{45}Ca^{2+}$ uptake in

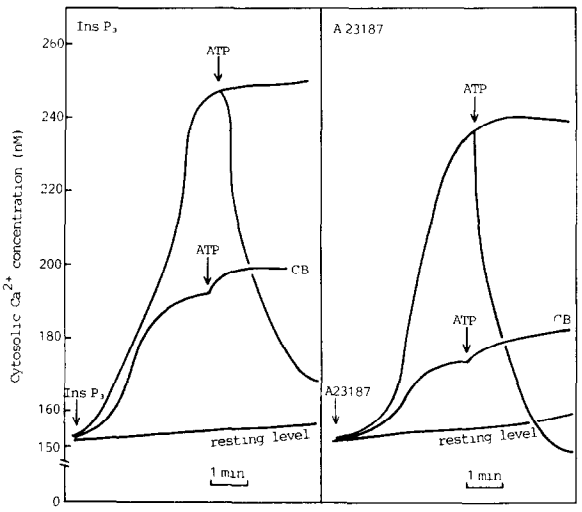

Fig.2. Inhibitory effects of cytochalasin B (CB) on either Ca^{2+} released by Ins P_3 (or A23187) or Ca^{2+} re-taken up by ATP in digitonin-permeabilized parietal cells as measured by quin2/AM. The concentrations of reagents tested were as follows; ATP, 1.5 mM; Ins P_3, 5 μM; A23187, 5 μg/ml; cytochalasin B(CB), 10 μg/ml. The data represent the means ± SE of 9 determinations from 3 separate experiments.

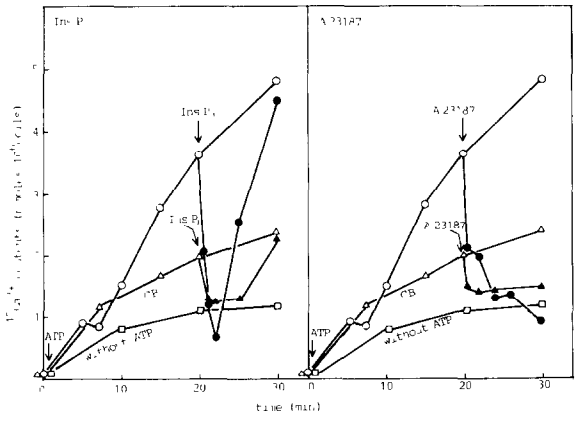

Fig.3. $^{45}Ca^{2+}$ released by either Ins P_3 or A23187 and its inhibitory effect due to cytochalasin B (CB) that are accumulated by ATP in digitonin-permeabilized parietal cells. The concentrations of reagents tested were as follows; ATP, 1.5 mM (○,△); Ins P_3, 5 μM (●,▲ left); A23187, 5 μg/ml (●,▲ right); cytochalasin B (CB), 10 μg/ml (△); without ATP (□). The data represent the means of duplicate determinations from 2 separate experiments.

digitonin-permeabilized cells. There was a rapid uptake of $^{45}Ca^{2+}$ following the addition of ATP (1.5 mM). The ATP-dependent $^{45}Ca^{2+}$ uptake at 20 min was 2.53 nmol/10^6 cells (with ATP, 3.63 nmol/10^6 cells, $n = 2$; without ATP, 1.10 nmol/10^6 cells, $n = 2$). When Ins P_3 (5 μM) or A23187 (5 μg/ml) was added, an enormous loss of $^{45}Ca^{2+}$ content occurred during the first 2 min. This $^{45}Ca^{2+}$ was accumulated into the pool by ATP. A consecutive rapid $^{45}Ca^{2+}$re-uptake (by ATP-regenerating system) when Ca^{2+} was released

Table 1

A23187-induced amino[^{14}C]pyrine accumulation and its inhibitory effect by cytochalasin B in intact parietal cells

Agents added	Amino[^{14}C]pyrine ratio (R)	
	Medium Ca^{2+}: Absence (Ca^{2+}, 0 mM; EDTA, 1 mM)	Presence (Ca^{2+}, 1.3 mM)
Control	10.40 ± 0.47	10.44 ± 0.23
A23187 (5 μg/ml)	25.83 ± 3.83[a]	24.03 ± 1.17[b]
A23187 (5 μg/ml) + cytochalasin B (10 μg/ml)	13.43 ± 0.34	29.21 ± 0.53[b]

The amino[^{14}C]pyrine trapped in the cell was expressed as a ratio using the formula by Berglindh [23].

$$\text{Ratio percentage } (R) = \frac{1 + 10^{(pK_a - \text{pH intracell})}}{1 + 10^{(pK_a - \text{pH medium})}} = \frac{\text{filter cpm} \times 100}{(2\ \mu\text{l/mg})\ (\text{mg dry wt})\ (\text{medium cpm}/3\ \mu\text{l})}$$

where the medium pH = 7.4 and the pK_a of aminopyrine = 5. The dry cell weight was multiplied by a factor of 2 μl/mg of the total cellular water. The dry weight of 10^6 parietal cells was 4.5 mg. 'Medium cpm/3 μl' stands for the radioactivity equaling the volume of 10^6 parietal cells (diameter of parietal cell = 18 μm, volume of 10^6 parietal cells = $4/3\ \pi(\text{radius})^3 \times 10^6 \doteqdot 3 \times 10^{-9}$ m^3 v.s. 1 ml of incubation medium = 10^{-6} m^3). R obtained in this study is reasonable since the volume of the acid compartment was estimated to be 1-4% of the total cellular volume [23]. Therefore, the pH in the acid compartment when A23187 was added was 1.5-2.0 when it was calculated from R. The data represent the mean ± SE from 3 to 6 separate experiments. The significant difference was calculated from the corresponding value ([a] control without medium Ca^{2+}; [b] control with medium Ca^{2+}) ($P < 0.05$) by the unpaired t-test.

by Ins P_3 but not by A23187 was observed. The permeable cells that were pretreated with cytochalasin B failed to accumulate the $^{45}Ca^{2+}$ induced by ATP (reduced by 45.0% at 20 min) and also failed to release the $^{45}Ca^{2+}$ induced by either Ins P_3 (reduced by 75.2%) or A23187 (reduced by 64.2%) within the first 2 min.

As shown in table 1, the early acid secretion evoked by A23187 at 10 min occurred regardless of the presence or absence of medium Ca^{2+} when amino[^{14}C]pyrine accumulation was monitored in intact parietal cells. The inhibitory effect of A23187-induced acid secretion by cytochalasin B was observed in the absence of medium Ca^{2+} but not in the presence of medium Ca^{2+}.

4. DISCUSSION

Gastrin is believed to be the first messenger of intracellular Ca^{2+}-mobilizing hormone in the parietal cell [6]. However, it is unclear how the cascade reaction after receptor occupancy (by gastrin) mobilizes intracellular Ca^{2+} and acid secretion. In addition, the characterization of an unidentified pool that releases and takes up Ca^{2+} has not been substantiated. This study, therefore elucidates the mechanism of intracellular Ca^{2+} metabolism of the parietal cell.

Digitonin (as well as saponin) is known to permeabilize the plasma membrane, but not mitochondria or endoplasmic reticulum, by forming a complex with cholesterol [26]. In digitonin-permeabilized parietal cells, Ins P_3 or A23187 released Ca^{2+} from an unidentified Ca^{2+} pool and Ca^{2+} released into the cytosol was then re-taken up by ATP into the same or similar pool. The rapid re-uptake of Ca^{2+} (by ATP) rather than its removal (by ATP) when cytosolic Ca^{2+} concentration is maintained at the resting level may be caused by the consequent activation of the Ca^{2+} pump due to the rise of cytosolic Ca^{2+} concentration (by Ins P_3). However, Ca^{2+} released by A23187 failed to be re-taken up by the ATP-regenerating system in contrast to that by consecutive addition of ATP (figs 1-3). This may be due to the increase of ATP hydrolysis because Ca^{2+} recycling across the membrane by A23187 allows the ATPase to maintain a higher rate of ATP hydrolysis since no Ca^{2+} gradient is formed.

The unidentified Ins P_3-sensitive and ATP-dependent Ca^{2+} pool is located in the non-mitochondrial fraction because of its insensitivity to 2,4-dinitrophenol (mitochondrial uncoupler) [17]. The quantity of Ca^{2+} taken up by ATP in permeable cells (2.53 fmol/cell per 20 min) was not much different from that taken by smooth surfaced membrane vesicles (1.22 ± 0.22 fmol/cell per 20 min, $n = 18$), but not by plasmalemma (14.25 ± 1.06 fmol/cell per 20 min, $n = 4$) or mitochondria (4.64 ± 0.06 fmol/cell per 20 min, $n = 16$), which were prepared by a sucrose density gradient (not shown). Treatment of permeable cells or intact cells with cytochalasin B caused inhibitions of ATP-dependent Ca^{2+} uptake (and/or re-uptake), Ins P_3 (or A23187)-induced Ca^{2+} release and A23187-induced acid secretion. Similar inhibitory effects of cytochalasin B on gastrin-elicited Ca^{2+} release and concomitant acid secretion has also been demonstrated [6]. The concentration of cytochalasin B used (10 μg/ml) is appropriate since it abolishes acid secretion induced by gastrin [6] or histamine [7] and enhances insulin secretion [27,28]. Besides disrupting microfilaments, cytotoxic effects such as inhibition of glucose transport and membrane protein rearrangement by cytochalasin B in this dose range [29,30], should be neglected since the inhibitory effects of cytochalasin B on acid secretion were not obviated when acetate was used as the substrate or dibutyryl cyclic AMP as a secretagogue [7,31]. In addition, the inhibitory effect of cytochalasin B on A23187-induced acid secretion was not observed when medium Ca^{2+} was present. A similar effect has been obtained that inhibitory effects of colchicine on prolactin and growth hormone released from pituitary cells required the absence of medium Ca^{2+} [32]. Therefore, it is unlikely that the observed effect is due to its cytotoxic effects. That is, the inhibitory effects of cytochalasin B on intracellular Ca^{2+} metabolism and acid secretion that are brought about by Ins P_3, A23187 and ATP may be due to the disruption or collapse of microfilaments. Cytochalasin B failed to inhibit the ATP-dependent Ca^{2+} uptake of subcellular vesicles [5], suggesting that its inhibitory effect requires cytosolic elements in intact cells. On the other hand, a previous study [5] revealed that lanthanum failed to inhibit the increase of cytosolic Ca^{2+} concentration by A23187 that was independent of medium Ca^{2+}. Lanthanum can penetrate

the secretory canaliculus, although it fails to penetrate the tubulovesicle [33], suggesting that the ATP-dependent and Ins P_3 (or A23187)-sensitive Ca^{2+} pool is in the cytoplasm but not secretory canaliculi. Microfilaments penetrate deeply into the cytoplasm, but no filaments consist of the associated tubulovesicular elements. However, these filaments form a meshwork between the tubulovesicular system and the plasma membrane just beneath the apical surface [8]. In addition, it is possible that phosphatidylinositol plays a part in the microtubular (and/or microfilaments)-plasmalemmal linkage [34]. Therefore, it seems that the Ins P_3-sensitive and ATP-dependent Ca^{2+} pool of the parietal cell is closely located in the apical surface and linked with microfilaments. Its function and concomitant acid secretion are regulated by the dynamic function of the microfilamentous system.

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