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Participation of the microtubular-microfilamentous system on intracellular Ca^{2+} transport and acid secretion in dispersed parietal cells

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Biphasic responses of amino[^{14}C]pyrine accumulation and oxygen consumption were registered by gastrin stimulation in dispersed parietal cells from guinea pig gastric mucosa, and this was mimicked with the calcium ionophore A23187. The characteristics of these phases (first phase and second phase) were distinguished by the differences in the requirements of extracellular Ca^{2+}. The first phase evoked by gastrin or ionophore A23187 was independent of extracellular Ca^{2+}, whereas the second phase was not. In the first phase, fluorescence of a cytosolic Ca^{2+} indicator (quin2-AM) increased with the stimulation of ionophore A23187 and carbamylcholine chloride in the presence of extracellular Ca^{2+}. In addition, an increase in cytosolic Ca^{2+} induced by ionophore A23187, but not by carbamylcholine chloride was also observed in the absence of extracellular Ca^{2+}, suggesting that Ca^{2+} pool(s) in parietal cells might be present in the intracellular organelle. Cytochalasin B and colchicine, but not oligomycin, could eliminate this cytosolic Ca^{2+} increase induced by A23187 in a Ca^{2+}-free medium. On the other hand, in a Ca^{2+}-free medium, addition of ATP after pretreatment with digitonin could diminish the cytosolic Ca^{2+} increase brought about by A23187. This was also observed with oligomycin-treated cells, but not with cytochalasin B-treated cells. Similarly, subcellular fractionation of a parietal cell which had been pretreated with cytochalasin B or colchicine in an intact cell system reduced the rate of ATP-dependent Ca^{2+} uptake. These observations indicate that intracellular Ca^{2+} transport in dispersed parietal cells may be regulated by the microtubular-microfilamentous system. In conclusion, this study demonstrates the possibility of the existence of intracellular Ca^{2+} transport mediated by gastrin or ionophore A23187 and regulated by the microtubular-microfilamentous system in parietal cells.

Introduction

Cyclic AMP and Ca^{2+} are thought to be second-stage messengers of the stimulation of acid secretion [1]. It may be concluded that cyclic AMP is a necessary intermediate between histamine and acid secretion [2]. Little is known, however, about the importance of Ca^{2+} for acid secretion, although the role of Ca^{2+} as an intracellular second messenger for excitation-contraction coupling or stimulus-secretion coupling in such structures as the sarcoplasmic reticulum [3], pancreatic acini [4] and the B-cell [5] has been examined in detail. In general, the muscarinic type of cholinergic receptor increases Ca^{2+} influx. Soll [6] and Berglindh et al. [7] have already proposed this principle in the case of the parietal cell, but it is still unknown

Abbreviations: Quin2-AM, 2-[2-amino-5-methylphenoxy]methyl-6-methoxy-8-aminoquinoline-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

whether Ca^{2+} is a second-stage messenger of cholinergic stimulation. Alternatively, Soll [6] has proposed the possibility that gastrin may act as a mobilizer of intracellular Ca^{2+} in one way or another. In this study, the relationship between intra- or extracellular Ca^{2+} mobilization and acid secretion by ionophore A23187 or gastrin has been investigated by using loading by quin2-AM (which is a new fluorescent, selective cytosolic Ca^{2+} indicator [8]), amino[^{14}C]pyrine accumulation [9] and oxygen consumption [10]. Microtubular-microfilamentous systems, including tubulovesicular elements and secretory canaliculi, participate in the gastric acid secretion process [11–13]. The cytochalasins are known to be disrupting agents of microfilaments in stimulus-secretion coupling model adapted cells [14] and have been shown to interact with actin [15]. Cytochalasin B has been reported to cause inhibition of acid secretion in piglet gastric mucosa by the disruption of microfilaments consisting of an actin-like protein linked with meromyosine [16,17]. Similarly, the inhibition of acid secretion by colchicine has been reported by Kasbekar et al. [18] and the colchicine-binding site was found in the $100\,000 \times g$ soluble fraction corresponding to tubulin in the 55 kDa region in bullfrog stomach [19]. In order to study possible interactions of intracellular Ca^{2+} transport and the microtubular-microfilamentous system, the effects of anticytoskeletal agents such as cytochalasin B and colchicine were examined with respect to intracellular Ca^{2+} mobilization in dispersed guinea pig parietal cells.

Materials and Methods

Agents. The following chemicals were obtained from the indicated sources. Ionophore A23187, human gastrin-17, carbamylcholine chloride, oligomycin, cytochalasin B, vincristine, lanthanum, dithiothreitol, ATP and collagenase type I (150 U/mg *Clostridium histolyticum*) were from Sigma, U.S.A. Colchicine and digitonin were from Nakarai Kagaku, Japan. Quin2-AM was from Dougin Kagaku, Japan. Dispase II (300000 proteinase U/g, *Bacillus polymixa*) was from Godo Shusei, Japan. Tissue culture medium (medium 199) was from Nissui Kagaku, Japan. Fetal calf serum and penicillin-streptomycin were from Gibco, U.S.A. Aprotinin (Trasylol®) was from Beohringer-Mannheim, F.R.G. Amino[^{14}C]pyrine dimethylamine (110 mCi/mmol), $^{45}Ca^{2+}$ (29.33 mCi/mg) and Aquasol were from New England Nuclear, U.S.A.

Isolation of parietal cells. Parietal cells were prepared from the gastric mucosa of nonfasted young guinea pigs (Hartley, male, weighing 250 g) by modification of the method previously described by Soll [10]. The mucosal strips removed from one guinea pig were minced with ophthalmologic scissors, for 2.5 min at 4°C in medium 199 (tissue culture medium which had been modified by Earle's medium). Total mucosal fragments were placed into 12.5 ml of medium 199, containing collagenase type I (0.1 mg/ml), dispase II (0.8 mg/ml), penicillin-streptomycin (100 U/ml) and 10% fetal calf serum, heat-inactivated (at a 56°C, for 30 min). This was incubated three times in a wide bottom plastic bottle, gassed through with air/CO_2 (95 : 5) at 37°C for 20 min in a shaker bath at 120 oscillations/min. At the end of each incubation period, the fragments were centrifuged at $100 \times g$ for 5 min, and the supernatant was discarded. The cell pellet from the final procedure was solubilized in 20 ml of Ca^{2+}- Mg^{2+}-free Krebs-Ringer bicarbonate solution containing 0.5 mM dithiothreitol (if not otherwise noted, it consisted of 122 mM NaCl/4.8 mM KCl/1.2 mM KH_2PO_4/25 mM $NaHCO_3$/0.2% glucose/0.2% bovine serum albumin/1.0 mM EDTA/10 mM Hepes (pH 7.4)) and was incubated for 20 min in a shaker bath at 120 oscillations/min at 37°C, gassed through with air/CO_2 (95 : 5). Pipetting was repeated 20 times to scatter cell clumps. The suspension was centrifuged at $10 \times g$ for 2 min and the supernatant was pooled (parietal cell enriched fraction). All pooled supernatant fractions were centrifuged at $200 \times g$ for 10 min, rinsed twice with medium 199 and passed through wire netting of 200 and 400 mesh. Viability, determined by exclusion of 0.4% Trypan blue, was almost 90% and the yield of parietal cells was almost 10^7 per one guinea pig gastric mucosa. The population of parietal cells was almost 60%. The population of parietal cells when further cell separation had been accomplished using a Beckman elutriator rotor [10] was 80%.

Measurement of free cytosolic Ca^{2+}. Quin2-AM (100 μM) was added to parietal cell enriched frac-

tions ($5 \cdot 10^6$ parietal cells/ml) in medium 199 containing 20 mM Hepes (pH 7.4) and was incubated for 20 min at 37°C, then subsequently diluted 5-fold with the same solution in order to lower the ester concentration and incubated for 40 min at 37°C, gassed through with air/CO_2(95 : 5). After loading, the cell suspension was rinsed twice at $100 \times g$ for 5 min and resuspended in fresh Krebs-Ringer bicarbonate solution (if not otherwise noted, it consisted of 122 mM NaCl/4.8 mM KCl/2.6 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/25 mM $NaHCO_3$/0.2% glucose/0.2% bovine serum albumin/10 mM Hepes (pH 7.4)) at about 10^6 parietal cells per 2 ml and secretagogues were added at the start of the experiment. Incubation was performed at 24°C for 15 min under stirring with an immunorotor. Quin2-AM fluorescence was recorded with a Hitachi 650-10 LC fluorescence spectrometer in cuvettes. The excitation and emission wavelengths were 339 and 492 nm with 4 and 10 nm bandwidths.

Amino[^{14}C]pyrine accumulation. The incubation medium contained 10^6 parietal cells plus the indicated agents and 1 ml of the total volume was adjusted with air/CO_2-saturated Krebs-Ringer respiratory medium. The incubation medium contained Trasylol (200 U/ml of the kallikrein inhibitor) for gastrin stimulation, including oxygen consumption analysis. Amino[^{14}C]pyrine (0.1 μCi) was added. The incubation medium without cells was preincubated for 30 min in a 95% O_2/5% CO_2 atmosphere at 37°C. 1 ml of each incubation medium was incubated for various time intervals at 28 rpm in the immunorotor at 37°C. At the end of the incubation period, a 700 μl cell suspension was placed directly on a Millipore filter (RAWP 1.2 μm) and immediately washed four times with 3 ml ice-cold 'stop solution', which is essentially the same as the incubation solution. Millipore filters had been rinsed previously overnight with 10% bovine serum albumin solution. Aspiration pressure was maintained at less than 125 mmHg and the aspiration time was less than 1 min, to avoid cell lysis. The dried filter was digested with 8 ml Aquasol and counted in a Packard liquid scintillation spectrometer. The amino[^{14}C]pyrine trapped in the cell was calculated as the percentage of the total amino[^{14}C]pyrine present in the parietal cells plus the medium.

Oxygen consumption analysis. The respiration was measured by a YSI 53 type oxygen analyzer. The methods are described elsewhere [10]. 1 ml of the cell suspension ($2 \cdot 10^6$ parietal cells) was added to 3 ml of air/CO_2 (95 : 5) saturated Krebs-Ringer respiratory medium in a chamber of the instrument. Oxygen consumption was measured and calculated from the percentage of the disappearance of oxygen per min and per $2 \cdot 10^6$ cells.

Measurement of $^{45}Ca^{2+}$ uptake activity. ATP-promoted $^{45}Ca^{2+}$ uptake studies of crude microsomal fractions in purified parietal cells are described in Table V and Fig. 4. In this experimental system, further cell separation was accomplished using a Beckman elutriator rotor [10], to avoid an involvement of ATP-dependent microsomal $^{45}Ca^{2+}$ uptake derived from cheif cells.

Results

Fig. 1 shows the gastrin-induced biological response investigated by amino[^{14}C]pyrine, which is an index of acid secretion in vitro. Amino-

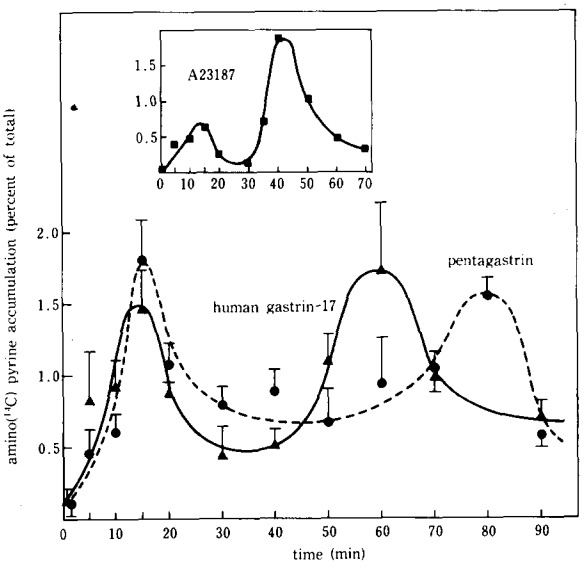

Fig. 1. Time-course of amino[^{14}C]pyrine accumulation stimulated with gastrin from dispersed parietal cells. 1 ml of the incubation medium contains 10^6 parietal cells plus indicated agents (human gastrin-17: 1 μM, pentagastrin: 3.25 μM, A23187: 5 μg/ml). The non-stimulated value was 0.468 ± 0.021 ($n = 15$, percent of total). Data represent seven separate experiments. Each point and vertical bar represents the mean $\pm$ S.E.

[^{14}C]pyrine accumulation of parietal cell stimulated with gastrin (pentagastrin or human gastrin-17) was biphasic. The pentagastrin-induced first peak occurred at 15 min (percentage of total, 1.78 ± 0.26, $n = 9$) and fell to 64% after 15 min (percentage of total, 0.78 ± 0.13, $n = 11$). A delayed peak of amino[^{14}C]pyrine accumulation response to pentagastrin was seen again at 80 min (percentage of the total, 1.56 ± 0.11, $n = 9$). The pattern with human gastrin-17-induced accumulation was similar. Ionophore A23187 mimicked these gastrin-induced biphasic responses on a small scale (Fig. 1 inset), suggesting that gastrin-induced acid secretion might have been mediated by Ca^{2+}. These biphasic amino[^{14}C]pyrine accumulations were different from the carbamylcholine chloride-elicited transient response and the histamine-elicited monophasic response [7,20]. Dibutyryl cyclic AMP-induced amino[^{14}C]pyrine accumulation was also monophasic (maximal accumulation 1.44 ± 0.36, percentage of total, $n = 6$), and this was inhibited neither by cimetidine (percentage of total, 1.59 ± 0.17, $n = 3$) nor by atropine (percentage of total, 1.21 ± 0.23, $n = 3$) (data not shown), indicating mode of action different from that of gastrin.

The maximal amount of amino[^{14}C]pyrine accumulated per parietal cell under gastrin or A23187 stimulation was almost 18 fmol, which equals the ouabain-insensitive K^+ uptake due to gastrin [21], suggesting that the H^+ and K^+ exchange occurs at a ratio of 1 : 1. Biphasic behavior was also observed when oxygen consumption was monitored (Fig. 2). Maximal oxygen consumption under stimulation with pentagastrin was manifestly biphasic. The first response was observed at 10–15 min and the latter at 70–80 min. The value of oxygen consumption corresponding to each time period was 16.9% ($\Delta O_2 = 0.766\ \mu l \cdot min^{-1} \cdot ml^{-1}$) and 12.5% ($\Delta O_2 = 0.566\ \mu l \cdot min^{-1} \cdot ml^{-1}$), respectively.

The amounts of oxygen consumption were approximate numbers from other reports [9,29,30] (Fig. 2a). Gastrin increased oxygen consumption to about 4.39- and 3.25-fold of the basal value in the first and second peak, respectively. The oxygen consumption at the intervals 15–70 min, corresponding to the minimal accumulation of amino[^{14}C]pyrine, was low and almost equal to the basal value. In contrast, carbachol- or histamine-induced increases in oxygen consumption were very quick and immediately reached a steady state

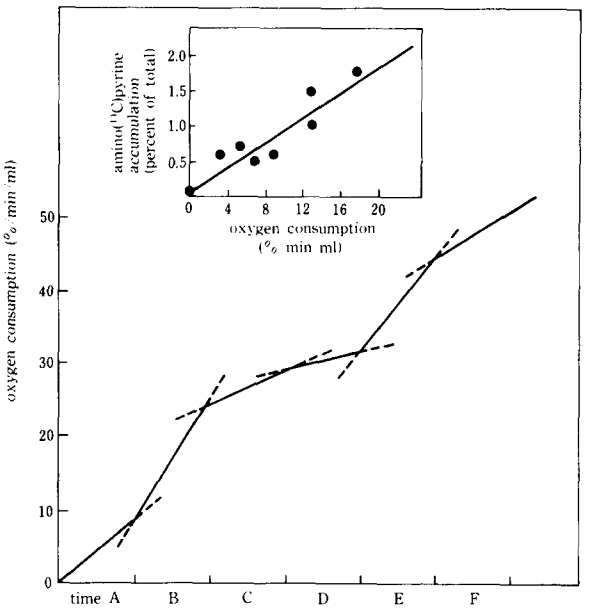

Fig. 2. Oxygen consumption pattern of pentagastrin in dispersed parietal cells. 1 ml of cell suspension ($2 \cdot 10^6$ cells) was incubated with pentagastrin (6.25 μM) at the following time intervals: A, 0–10; B, 10–15; C, 15–30; D, 30–70; E, 70–80; F, 80–90 min, respectively. At zero time of preincubation, cell suspension was immediately transferred to 3 ml Krebs-Ringer solutions in a chamber of YSI 53 TYPE oxygen analyzer. Maximal oxygen consumption by pentagastrin was manifested at 10–15 min (B) and 70–80 min (E) in which corresponding value was 16.9% (0.77 μl/min per ml) and 12.5% (0.57 μl/min per ml), respectively. The basal line indicates oxygen consumption without a stimulant, representing constant value (3.85%) which was almost equal to the value stimulated with pentagastrin for 15–70 min (C and D). Data represent three separate experiments. All weights in the following are dry weights. Inset: the data for stimulation of oxygen consumption by pentagastrin has been plotted as a function of the effect on amino[^{14}C]pyrine accumulation. The correlation coefficient for the relation was 0.879 ($P < 0.05$). (a) The calculation of this value is expressed as Δ $\mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$ as follows: first response, $0.766\ \mu l \cdot min^{-1} \cdot ml^{-1} \times 4$ ml (total volume) $\div 15$ mg (weight of total cell volume) $\times 0.6$ (paritetal cell population) $\times 60$ min $= 20.4\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$; second response, $0.566 \times 4 \div (15 \times 0.6) \times 60 = 15.1\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$; basal, $0.170 \times 4 \div (15 \times 0.6) \times 60 = 4.5\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$. The above calculated values are approximate numbers from other reports as follows: Chew et al. [29], histamine (100 μM), rabbit, $14\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$. Berglindh et al. [9], carbachol (140 μM), rabbit, $24\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$. Lewin et al. [32], basal, rat, 700 $nmol \cdot h \cdot 10^{-6}$ cells $\div 4.98\Delta\ \mu l\ O_2 \cdot mg^{-1} \cdot h^{-1}$.

(data not shown). Cell suspensions without secretagogue (basal) showed a linear consumption of oxygen (2.33–3.85%/min). The correlation coefficient (r) between oxygen consumption and amino[^{14}C]pyrine uptake stimulated by pentagastrin was 0.879 ($P < 0.05$) (Fig. 2, inset). The reaction of biphasic acid secretion by gastrin in vitro was combined with a morphological analysis. The appearance of numerous secretory canaliculi containing long microvilli and the disappearance of tubulovesicles were observed at 15 and 60 min after gastrin stimulation (data not shown). The reason for the dual acid secretion in vitro induced by gastrin and A23187 is not known in detail, but it can be further characterized by its requirement of extracellular Ca^{2+}.

The first phase of both stimulations was independent of extracellular Ca^{2+}, but in contrast, the second phase was completely dependent on extracellular Ca^{2+} (Table I). The first response induced by gastrin (independent of extracellular Ca^{2+}) was not inhibited by addition of lanthanum (100 μM) in a Ca^{2+}-containing medium (1.3 mM Ca^{2+}, 0.748 ± 0.108, $n = 9$, Ca^{2+}-free, 0.640 ± 0.171, $n = 6$, 1.3 mM Ca^{2+} + 100 μM La^{3+}, 0.850 ± 0.112, $n = 4$, $\Delta\mu l\ O_2 \cdot min^{-1} \cdot ml^{-1}$, respectively), which confirms its independence of extracellular Ca^{2+}. Therefore, extra- or intracellular Ca^{2+} mobilization in parietal cells was examined.

As shown in Table II, a quin2-AM loading study has revealed that A23187 did increase the cytosolic Ca^{2+} in a Ca^{2+}-containing medium, regardless of the K^+ concentration. Moreover, in a Na^+-free medium (replaced by choline$^+$ to maintain the ionic strength), A23187 enhanced the increase in cytosolic Ca^{2+}, but in a Ca^{2+}-free medium the increase in cytosolic Ca^{2+} by A23187 was not affected in the presence or absence of extracellular Na^+. In this conditioning medium (2.6 mM Ca^{2+}/108 mM K^+, Na^+-free), human gastrin-17 (1 μM) and carbamylcholine chloride (100 μM) were also able to enhance significantly the cytosolic Ca^{2+}

TABLE I

AMINO[^{14}C]PYRINE ACCUMULATION STIMULATED BY IONOPHORE A23187 AND OXYGEN CONSUMPTION STIMULATED BY GASTRIN IN THE PRESENCE OR ABSENCE OF Ca^{2+}

Amino[^{14}C]pyrine accumulation is expressed as a percentage of the total. Oxygen consumption was measured by a YSI oxygen analyzer and the quantity was expressed as $\Delta\mu l\ O_2 \cdot min^{-1} \cdot ml^{-1}$ per 10^6 parietal cells. The values of non-stimulated cell were as follows; 0.377 ± 0.064 (1.3 mM Ca^{2+}), 0.289 ± 0.037 (Ca^{2+}-free), $n = 5$, respectively. Each value represents mean ± S.E. of the number of samples in parentheses from three separate experiments. The significant difference was calculated from the corresponding control values, in the presence of Ca^{2+}, (* $P < 0.05$) using the unpaired t-test. The Ca^{2+}-free medium was prepared by omitting $CaCl_2$ and by adding 1.0 mM EDTA in Krebs-Ringer bicarbonate solution. In the Ca^{2+}-free experiments, Mg^{2+} in addition to Ca^{2+} was omitted from the medium (Tables I–IV and Fig. 3).

	First response	Second response
Amino[^{14}C]pyrine accumulation		
2.6 mM Ca^{2+}	0.524 ± 0.040(3)	1.082 ± 0.221 (5)
Ca^{2+}-free	0.618 ± 0.024(3)	0.507 ± 0.055*(4)
Oxygen consumption		
1.3 mM Ca^{2+}	0.748 ± 0.108(9)	1.014 ± 0.161 (6)
Ca^{2+}-free	0.642 ± 0.171(6)	0.257 ± 0.045*(3)

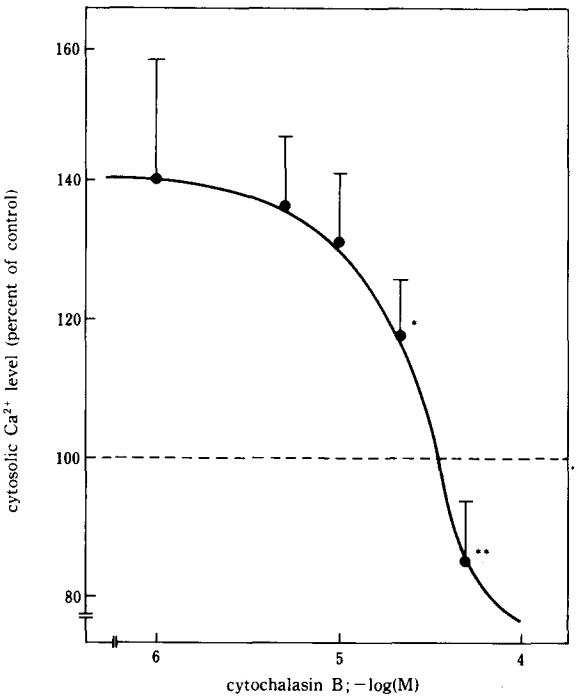

Fig. 3. Dose inhibition curve of cytochalasin B on A23187 (1 μg/ml)-induced cytosolic Ca^{2+} rise in a Ca^{2+}-free medium by quin2-AM loading. Each point and vertical bar represents the mean ± S.E. of five separate experiments. The significant difference from control values (A23187 alone) was: * $P < 0.05$, ** $P < 0.01$. 100% corresponds to the fluorescence in non-stimulated cells.

TABLE II

CYTOSOLIC Ca^{2+} LEVELS AND RESPONSES TO SECRETAGOGUES BY QUIN2-AM LOADING IN THE PRESENCE OR ABSENCE OF EXTRACELLULAR Ca^{2+}

A23187 and human gastrin-17 (HG-17) were dissolved in 20% dimethylsulfoxide, and this final concentration (0.1%) of dimethylsulfoxide had no effect on fluorescence and cellular function. A23187 gave rise to fluorescence itself and was subtracted from the observed value (the arbitrary unit is 30 of 5 μg/ml A23187 if the value of quin2-AM-loaded non-stimulated cells are 20). Quin2-AM was dissolved in 60% dimethylsulfoxide and gross precipitates were dispersed by sonication. Each value (percent of control) represents mean ± S.E. of 5–7 separate experiments. 100% corresponds to the fluorescence in non-stimulated cells. Cytosolic Ca^{2+} levels of non-stimulated cells were almost equal in many constituting media. The significant difference was calculated from control values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) using the paired t-test.

Constitution of $Ca^{2+}/Na^{+}/K^{+}$ (mM)	Agents added		Cytosolic Ca^{2+} level (percent of control in arbitrary units)
2.6/122/4.8	A23187	0.83 μg/ml	214.1 ± 27.9 *
		2.50	280.9 ± 36.7 *
		5.00	299.8 ± 39.2 *
		8.33	479.6 ± 62.1 **
	HG-17	1 μM	104.9 ± 9.5
2.6/40.6/108	A23187	5.00 μg/ml	271.5 ± 12.8 ***
	HG-17	1 μM	130.1 ± 7.8 *
2.6/0/108 [a]	A23187	5.00 μg/ml	361.5 ± 31.3 **
	HG-17	1 μM	216.0 ± 3.0 *
	carbamylcholine chloride	100 μM	195.6 ± 2.0 *
0/122/4.8	A23187	5.00 μg/ml	168.6 ± 3.5 *
0/40.6/108	A23187	5.00 μg/ml	166.4 ± 3.0 ***
0/0/108 [a]	A23187	5.00 μg/ml	173.0 ± 6.0 *
	carbamylcholine chloride	100 μM	83.3 ± 6.2

[a] Na^{+} was substituted to 40.6 mM choline chloride.

increase. In a Ca^{2+}-containing medium, A23187 and carbamylcholine chloride significantly enhanced the increase in cytosolic Ca^{2+}, but replacement of extracellular Ca^{2+} by 1.0 mM EDTA failed to prevent the cytosolic Ca^{2+} increase induced by A23187, but not that induced by carbamylcholine chloride (Table II, middle and below). Arbitrary units of quin2-AM fluorescence in non-stimulated parietal cell (control) were almost the same in the presence or absence of extracellular Ca^{2+}. The increase in cytosolic Ca^{2+} induced by A23187 in a Ca^{2+}-free medium was about half that occurring in the presence of extracellular Ca^{2+}. This reduced A23187-induced cytosolic Ca^{2+} increase in a Ca^{2+}-free medium suggests that A23187 acts on the liberation of Ca^{2+} from an intracellular organelle, because the action of carbamylcholine chloride was based mainly on the external influx of Ca^{2+} [6]. The action of gastrin in a Ca^{2+}-free medium has not been examined in this study. Table III shows the effects of microtubular-microfilamentous disrupting agents (cytochalasin B, colchicine and vincristine), a mitochondrial poison (oligomycin) and lanthanum on the A23187-induced increase in cytosolic Ca^{2+} in a Ca^{2+}-free medium.

Cytochalasin B and colchicine, but not vincristine, oligomycin and lanthanum, significantly eliminated this cytosolic Ca^{2+} rise, thus suggesting that release of Ca^{2+} from intracellular Ca^{2+} pool(s) might be regulated by the microtubular-microfilamentous system. The dose-response curve for the effect of cytochalasin B on the increase in cytosolic Ca^{2+} induced by A23187 in a Ca^{2+}-free

TABLE III

EFFECTS OF MICROTUBULAR-MICROFILAMENTOUS SYSTEM DISRUPTING AGENTS, MITOCHONDRIAL POISON AND La^{3+} ON A23187-INDUCED INTRACELLULAR Ca^{2+} RISE BY QUIN2-AM LOADING IN A Ca^{2+}-FREE MEDIUM

Ionophore A23187 was present at 5 μg/ml (except in control). Each value represents mean ± S.E. of the number of samples in parentheses from four separate experiments, conditioning with Na^+/K^+ : 122/4.8 mM. 100% corresponds to the fluorescence in non-stimulated cells. The significant difference from A23187 alone (* $P < 0.05$) was calculated using the unpaired t-test from fluorescent arbitrary units.

Agent added	Cytosolic Ca^{2+} level (percent of control in arbitrary units)
Control	100 (9)
A23187 (5 μg/ml)	200.1 ± 6.2 (36)
+ cytochalasin B (10 μg/ml)	143.0 ± 13.5* (9)
+ colchicine (10 μg/ml)	126.2 ± 7.9* (9)
+ vincristine (10 μg/ml)	208.6 ± 12.8 (9)
+ oligomycin (5 μg/ml)	209.9 ± 13.6 (9)
+ lanthanum (0.1 mM)	199.3 ± 12.4 (9)

TABLE IV

EFFECTS OF ATP ON A23187 (5 μg/ml)-INDUCED CYTOSOLIC Ca^{2+} LEVEL IN A Ca^{2+}-FREE MEDIUM BY QUIN2-AM LOADING

Quin2-AM-loaded and digitonin- (20 μg/ml) pretreated cell suspension (containing 10^6 parietal cells) was incubated for 15 min at 24°C with 1 mM ATP (+) or without (−), plus the indicated agents. Each value represents mean ± S.E. of the number of samples in parentheses from four separate experiments. 100% corresponds to the fluorescence in non-stimulated cells (digitonin alone). The significant difference was calculated from the corresponding control values (ATP absence), (* $P < 0.05$, ** $P < 0.01$) using the unpaired t-test calculated from fluorescent arbitrary unit. In this experiment, the value of cytosolic Ca^{2+} level in A23187 alone was 150.4 ± 14.8% (9).

Agents added	ATP	Cytosolic Ca^{2+} level (percent of control in arbitrary units)
Digitonin	−	100 (4)
	+	87.1 ± 4.7*(4)
A23187 + digitonin	−	133.3 ± 13.2 (4)
	+	108.8 ± 9.8*(4)
A23187 + digitonin + cytochalasin B (10 μg/ml)	−	114.1 ± 7.6 (5)
	+	118.3 ± 11.2 (3)
A23187 + digitonin + oligomycin (5 μg/ml)	−	146.6 ± 24.7 (4)
	+	98.5 ± 14.2*(4)

medium is shown in Fig. 3. The IC_{50} of cytochalasin B is approx. 10^{-5} M. This dose range of cytochalasin B might be appropriate because acid secretion was partially or completely abolished at $1 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M cytochalasin B [16]. The inability of lanthanum to affect cytosolic Ca^{2+} might be due to its failure to penetrate the tubulovesicular system, although such penetration has been demonstrated in microvilli [12]. Alternatively, dispersed parietal cells which were permeable against ATP by digitonin pretreatment partially failed to increase the cytosolic Ca^{2+} concentration in response to A23187 in a Ca^{2+}-free medium, and even decreased the cytosolic Ca^{2+} level. After digitonin treatment, A23187 alone and A23187 plus oligomycin, but not A23187 plus cytochalasin B (when ATP was added), decreased the Ca^{2+} level compared with the value in the absence of ATP (Table IV). This indicates that an ATP-energized Ca^{2+} uptake mechanism may exist in parietal cell.

To determine the subcellular origin of the vesicles accumulating Ca^{2+}, we examined some characteristics of ATP-dependent $^{45}Ca^{2+}$ uptake in a cell-free system. In the experiment shown in Table V, ATP- and Mg^{2+}-dependent Ca^{2+} uptake was found in the crude microsomal fraction of purified parietal cells using a Beckman elutriator rotor. At the same time, microsomal vesicles were initially loaded with $^{45}Ca^{2+}$ by ATP, then uptake was stopped by EGTA (1 mM) alone or EGTA plus A23187 (5 μg/ml). A23187 induced a rapid release of $^{45}Ca^{2+}$ from microsomes, suggesting that the accumulated Ca^{2+} exists in an internal store of vesicles and is not bound to their exterior. In this system, addition of the cytochalasin B or colchicine directly to the microsomal fraction did not have any effect. In contrast, as shown in Fig. 4, the microsomal fraction obtained from cytochalasin B- or colchicine-pretreated parietal cells had a reduced rate of ATP-dependent $^{45}Ca^{2+}$ uptake. The inhibitory effect of cytochalasin B or colchicine on ATP-dependent microsomal Ca^{2+} up-

TABLE V

Ca^{2+} UPTAKE IN MICROSOMES FROM PARIETAL CELLS

The parietal cell-enriched fraction was loaded with flow rate of 48 ml/min at a centrifugal speed of between 1460 and 2000 rpm using a Beckman elutriator rotor. Portions of the cell fraction (parietal cell, more than 80%) were dissolved with 2 ml of 0.32 M sucrose, then homogenized and centrifuged at 5000 rpm for 20 min. The $2000 \times g$ supernatant was equilibrated with incubation buffer for 4 h, resulting in a final solution of the following composition; 100 mmol/l KCl, 4.5 mmol/l $MgCl_2$, 0.8 μmol/l $CaCl_2$, 20 mmol/l oxalate, 10 μg/ml oligomycin, 1.0 μCi/ml $^{45}Ca^{2+}$, in 50 mmol/l Tris-maleate (pH 7.4) in the presence or absence of cytochalasin B or colchicine. After 3 min of preincubation at 30°C, Tris-ATP was added at the final concentration of 1 mmol/l. After 20 min of the incubation periods, the reaction was stopped with 2 ml of ice-cold 'stop solution' containing 1 mM EGTA which was basically the same as the incubation medium. The medium was placed directly on a Millipore filter (0.3 μm pore size) which was rinsed with 1 M KCl and immediately washed four times with 2 ml of 'stop solution'. Radioactivity remaining on the filter was determined in a Packard liquid scintillation counter using the ^{14}C channel. The significant difference was calculated from the control values (5 mM ATP) (*** $P < 0.001$) using the unpaired *t*-test. Data represent mean ± S.E. of the number of samples in parentheses from four separate experiments.

Uptake	$^{45}Ca^{2+}$ content (nmol/mg protein per 20 min)	
5 mM ATP	30.0 ± 8.5	(10)
5 mM ATP in Mg^{2+}-free	5.7 ± 0.1***	(4)
Extrusion	$^{45}Ca^{2+}$ remaining (nmol/mg protein per 15 min)	
1 mM EGTA	30.9 ± 0.5	(6)
1 mM EGTA + 5 μg/ml A23187	3.1 ± 0.5***	(6)
Conditioning medium	$^{45}Ca^{2+}$ uptake (% of control)	
5 mM ATP	100	(6)
ATP-free	18.6 ± 1.25***	(4)
5 mM ATP in 0°C	13.2 ± 0.17***	(4)
5 mM ATP + cytochalasin B		
1.0 μg/ml	103.7 ± 4.65	(4)
2.5	104.6 ± 3.46	(4)
10.0	128.4 ± 6.30	(8)
5 mM ATP + colchicine		
1.0 μg/ml	125.6 ± 4.94	(4)
2.5	108.5 ± 8.06	(4)
10.0	136.9 ± 4.02	(8)

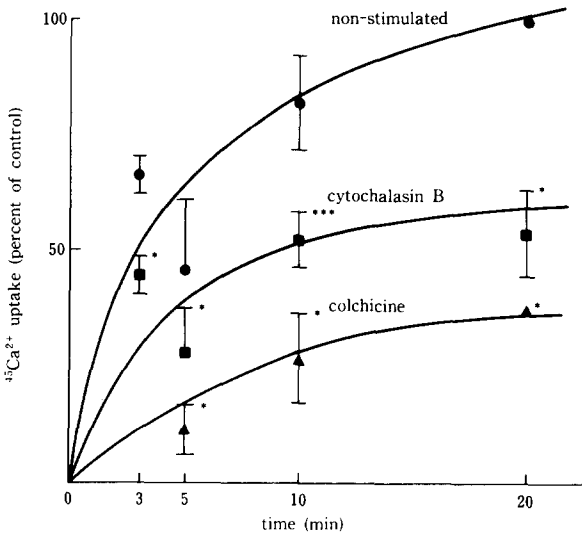

Fig. 4. Microsomal $^{45}Ca^{2+}$ uptake obtained from cytochalasin B- or colchicine-pretreated parietal cells. Parietal cells which were separated by a Beckman elutriator rotor were suspended with 2 ml of Krebs-Ringer solution, then incubated for 10 min at 30°C with or without cytochalasin B (10 μg/ml) and colchichine (10 μg/ml). Other methods are similar to those described in Table III. Each point and vertical bar represents the mean ± S.E. from the three typical experiments. The significant difference from the value corresponding to non-stimulated cells in each time period: * $P < 0.05$; *** $P < 0.001$.

take required intact cells. This supports the idea that intracellular ATP promotes Ca^{2+} uptake into a compartment whose function is modulated by the microtubular-microfilamentous system in the intact parietal cell.

Discussion

Parietal cells from guinea pig gastric mucosa were shown to be sensitive to gastrin stimulation as well as to other secretagogues such as carbamylcholine chloride, histamine and dibutyryl cyclic AMP [7,20]. The calcium ionophore A23187 mimics gastrin stimulation in showing a dual pattern with respect to aminopyrine accumulation. This observation suggests a similarity of these secretagogues in acid secretion. The first response evoked by gastrin or A23187 is independent of external Ca^{2+} concentration, but the second strongly requires extracellular Ca^{2+}, thus indicating the presence of two different mechanisms of

acid secretion, namely, that by intra- and that by extracellular Ca^{2+} mobilization. In this respect, Kasbekar [1] has already reported that the requirement for the acid secretion process can be differentiated into one for extracellular and one for intracellular Ca^{2+}. A similar role was proposed in glucose-stimulated release of insulin by the pancreatic B-cell [5], and cholecystokinin- or acetylcholine-stimulated release of amylase by the pancreatic acinar cell [4]. Therefore, the relationship of acid secretion and calcium mobilizaiton in parietal cells was examined. Quin2-AM can be taken to measure cytoplasmic free Ca^{2+} [8]. By using quin2-AM, it was shown that A23187 could enhance intracellular Ca^{2+} concentration, particularly in a Na^+-free medium. In a Na^+-free, high K^+ medium, A23187, carbamylcholine chloride and gastrin could also evoke increases in intracellular Ca^{2+}. Extracellular Na^+ may be inhibitory to cytosolic Ca^{2+} increases brought about by stimulation of secretagogues. This is due to the inhibition of Ca^{2+} influx, but not to the inhibition of intracellular Ca^{2+} release, because the increase in cytosolic Ca^{2+} brought about by A23187 in a Ca^{2+}-free medium occurred regardless of the presence of extracellular Na^+. It has been demonstrated that extracellular Na^+ was not essential for acid secretion [22,23] and cellular Na^+ could inhibit acid secretion [24]. The inhibition of acid secretion in the presence of a high concentration of Na^+ may be caused by a decline in the cytosolic Ca^{2+} increase. In the first step of acid secretion, A23187 could partially produce an increase in cytosolic Ca^{2+} in a Ca^{2+}-free medium with 1.5 mM EDTA, suggesting the existence of intracellular Ca^{2+} pool(s). In a Ca^{2+}-free medium, poisoning of mitochondria by oligomycin did not affect the increase in cytosolic Ca^{2+} induced by A23187, but alternatively, microtubular-microfilamentous poisons such as cytochalasin B and colchicine caused a dose-dependent inhibition of this cytosolic Ca^{2+} rise.

This suggests that intracellular Ca^{2+} transport in parietal cells may be regulated by the microtubular-microfilamentous system, but not by mitochondria. In similar way, it has been proposed that in the B-cell, microtubular-microfilamentous poisons such as cytochalasin B and vincristine modify insulin secretion by influencing the response to Ca^{2+} [25]. Additionally, in a Ca^{2+}-free, but not in a Ca^{2+}-containing medium, the addition of ATP to digitonin-pretreated parietal cells could avoid an increase in cytosolic Ca^{2+} in response to A23187, which phenomenon was observed with A23187 alone or in oligomycin- and A23187-treated cells, but not with cytochalasin B, suggesting that a part of Ca^{2+}-removal systems in the cell, that dependent on ATPase, is regulated by the microtubular-microfilamentous system. This proves that the crude microsomal fraction of parietal cell has the ability to perform ATP-promoted Ca^{2+} uptake. An inhibitory effect of cytochalasin B or colchicine was also found in this system. From the finding of the difference between the intact cell and cell-free systems in sensitivity to inhibition by microtubular-microfilamentous disrupting agents, one can consider the possibility that the microtubular-microfilamentous system may associate with other cellular components involved in intracellular Ca^{2+} transport. The inhibition of ATP-promoted Ca^{2+} uptake into crude microsomal vesicles stemming from intact parietal cells pretreated by cytochalasin B or colchicine may be due to suppression of the cytosolic Ca^{2+} rise, because the direct addition of these agents to microsomal vesicles did not result in any effect on Ca^{2+} uptake. Concerning the participation of intracellular Ca^{2+} mobilization in the onset of acid secretion, Soll [6] has proposed that gastrin may act as a mobilizer of Ca^{2+} from intracellular stores, thus being different from histamine, which produces cyclic AMP, and from cholinergic stimulations, which increases Ca^{2+} influx from the extracellular space [7]. Similarly, Jacobson et al. [26] have demonstrated that in vivo the first phase of acid secretion was partially reduced by removal of Ca^{2+}, in contrast to the second phase, suggesting that intracellular Ca^{2+} may participate in the early acid secretion. The findings of this present study seem to establish the fact that, in the first step of acid secretion by gastrin or A23187, intracellular Ca^{2+} release regulated by the microtubular-microfilamentous system occurs.

However, the importance of ATP-dependent Ca^{2+}-removing systems for either intracellular Ca^{2+} uptake or Ca^{2+} extrusion has not been assessed in the parietal cell. Recently, Muallem et al. [27] demonstrated the presence of a calmodulin-regu-

lated, ATP-dependent Ca^{2+} pump that could maintain active Ca^{2+} extrusion in the basal-lateral membrane of the parietal cell during cholinergic stimulation. The sensitivity to cytochalasin B or colchicine presented in this study may suggest that the ATP-dependent Ca^{2+}-removing system is derived from the apical surface and comprises tubulovesicular elements and the secretory canaliculus, as reported by Nandi et al. [28].

Characterization of the second acid secretion was not achieved in this study. But Kasbekar et al. [18] have reported that an initial but transient colchicine-mediated stimulation of acid secretion was followed by inhibition. This may suggest the possibility that the first acid secretion reflects the passage of accumulated H^+ from the tubulovesicles to the secretory canliculus and that this is due to mobilization of intracellular Ca^{2+}.

In conclusion, intracellular Ca^{2+} transport regulated by the microtubular-microfilamentous system may participate in the onset of acid secretion.

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