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Initial and sustained calcium mobilizations in the parietal cell during stimulations with gastrin, inositol trisphosphate, phorbol ester and exogenous diacylglycerol

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Electron probe X-ray microanalysis revealed that cytoplasmic Ca^{2+} concentration increased in the restricted apical cytoplasm during stimulation of isolated guinea pig parietal cells with gastrin. Furthermore, this study, using $^{45}Ca^{2+}$, aequorin and fura-2, revealed the mechanism involved in intracellular Ca^{2+} shifts caused by gastrin and the involvements of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol in producing those shifts. The gastrin-mediated and IP_3-sensitive Ca^{2+} pool was located in the smooth-surfaced membrane-enriched areas and released Ca^{2+} in the initial phase. Gastrin-mediated Ca^{2+} mobilization was also evoked by diacylglycerol, comprising an intracellular Ca^{2+} mobilization followed by a late, sustained and more localised Ca^{2+} entry from the extracellular space.

Ca^{2+} mobilization; Gastrin; Inositol trisphosphate; Diacyglycerol; (Guinea pig parietal cell)

1. INTRODUCTION

The parietal cell responds to endogenous mediators such as histamine via paracrine, acetylcholine via neurocrine and gastrin via endocrine. These stimulants act upon specific receptors (H_2 receptor for histamine, muscarinic cholinergic receptor for acetylcholine and gastrin receptor for gastrin) and produce separate second messengers (cyclic AMP for histamine, Ca^{2+} entry from the extracellular space for acetylcholine and intracellular Ca^{2+} release from the store(s) for gastrin) [1–3]. Among others, this study describes gastrin-elicited Ca^{2+} mobilization. Although it has not yet been substantiated that gastrin induces inositol trisphosphate (IP_3) production, an ATP-dependent and IP_3-sensitive Ca^{2+} pool connected with microfilaments is located in or near the apical portion of the parietal cells that comprises two types of membrane – tubulovesicular and canalicular membranes [4]. In addition, gastrin and IP_3 share common mechanism(s) in evoking intracellular Ca^{2+} release from the store(s) through the microtubule-microfilament regulated system [3,4]. Diacylglycerols substituted with long-chain fatty acids potentiate secretion synergistically with Ca^{2+} by activating protein kinase C [5]. Analogues of diacylglycerols and phorbol ester are known to be capable of activating protein kinase C [6–8]. Protein kinase C is also present in the parietal cell [9], and is probably involved in intracellular mechanisms regulating acid secretion evoked by gastrin [10,11]. Therefore, this study has been focused on defining Ca^{2+} mobilization in the parietal cell during stimulations with gastrin, IP_3, the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and the diacylglycerol 1-oleoyl-2-acetylglycerol (OAG). This problem has been ap-

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Abbreviations: fura-2, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid

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proached via a variety of techniques such as electron microprobing, the use of Ca^{2+}-indicating probes (aequorin and fura-2) and isotope $^{45}Ca^{2+}$ labelling, including some that employ the use of intact cells, plasmalemmal permeabilized cells or subcellular fractions.

2. EXPERIMENTAL

2.1. *Isolation of parietal cells*

Heterogeneous gastric mucosal cells from guinea pig gastric mucosa were prepared as in [12,13]. Enriched cell populations were prepared by Percoll density gradient ultracentrifugation (30000 × *g* for 15 min at 4°C). A fraction enriched with parietal cells was recovered at a density of between 1.043 and 1.050 g/ml [14,15].

2.2. *Electron probe X-ray microanalysis*

For electron probe X-ray microanalysis, parietal cells in the resting and stimulated (3 min after gastrin addition) states were placed on copper specimen holders and rapidly frozen by pressing against the wall of a copper block which had been precooled in liquid nitrogen. Cryosectioning of the specimens and electron probe X-ray microanalysis were carried out. Details of the procedures have been reported [16,17].

2.3. *$^{45}Ca^{2+}$ flux by permeable cells*

Isolated parietal cells (10^6 cells/ml) were immediately resuspended in a medium resembling the 'cytosol buffer' as in [4,13,14]. After permeabilization of cells with saponin (75 μg/ml) for 20 min at 37°C, the cells were resuspended in the 'cytosolic buffer' without saponin but with 1 mM EGTA, 0.49 mM $CaCl_2$, 1% bovine serum albumin, 1.0 μCi $^{45}Ca^{2+}$, 10 μM antimycin, 10 μM 2,4-dinitrophenol and an ATP-regenerating system (5 mM creatine phosphate, 50 μg/ml creatine phosphokinase). The medium Ca^{2+} concentration was set at 180 nM. After the addition of ATP, IP_3 or gastrin was added at 20 min. The final reaction was stopped by adding 2 ml of the same ice-cold medium without $^{45}Ca^{2+}$. The cell suspension was placed directly on a Millipore filter under mild suction and radioactivity remaining on the filter was counted as described [4].

2.4. *Subcellular fractionation*

Isolated parietal cells (10^7 cells) were homogenized at 0–4°C in 2 ml of 0.32 M sucrose buffer with 5 mM Tris-maleate (pH 7.4) in a Teflon-glass homogenizer and the smooth-surfaced membrane-enriched fraction was prepared as described in [13]. This fraction (100 μg protein) was suspended in an incubation buffer consisting of 100 mM KCl, 4.5 mM $MgSO_4$, 1.0 μM $CaCl_2$ prepared by Ca^{2+}-EGTA buffer, 20 mM oxalate and 1.0 μCi $^{45}Ca^{2+}$ in 5 mM Tris-maleate buffer (pH 7.2) in a final volume of 900 μl. Tris-ATP (100 μl) was added to give a final concentration of 1.5 mM and $^{45}Ca^{2+}$ remaining in the vesicles was separated by filtration [13].

2.5. *^{125}I-gastrin-binding assay*

A parietal cell suspension (10^6 cells) in 1 ml oxygenated Krebs-Ringer-bicarbonate solution (pH 7.4) was incubated with 0.05 μCi (23 pM) ^{125}I-labelled human gastrin-17 (spec. act. 2200 Ci/mmol, NEN, USA) for 15 min at 37°C. The cell suspension was then centrifuged at 10000 × *g* for 30 s to separate bound from free hormone and radioactivity in the resultant pellet was counted with a gamma counter (Alloka, USA). Specific binding was expressed as the value without isotope-free gastrin (total binding) minus that with excess unlabelled gastrin (100 nM) (non-specific binding). All data were fitted by least-squares regression from those of the dose-response curve of isotope-free gastrin (from 10^{-12} to 10^{-7} M) on specific ^{125}I-gastrin binding to parietal cells, the Scatchard plot being represented in fig.2.

2.6. *Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)*

The method of fura-2 loading has been previously described in gastric chief cells [14] and parietal cells [15]. Fluorescence was recorded with a Hitachi 650-60 fluorescence spectrophotometer equipped to allow for continuous stirring of the sample maintained at 37°C. Aequorin loading was performed according to [15,18] as a modification of the methods in [19,20]. Aequorin luminescence was determined with a platelet ionized calcium aggregometer (PICA) (Chrono Log, USA) equipped to allow for continuous stirring of the sample maintained at 37°C. $[Ca^{2+}]_i$ was calculated as in [21] by lysing cells using 0.1% Triton X-100 after incorporation of aequorin.

All chemicals and instruments used have been described in [3,4,12–18] unless otherwise stated.

3. RESULTS

3.1. *Ca^{2+} mobilization during gastrin stimulation (by electron probe X-ray microanalysis)*

Representative X-ray spectra from resting and gastrin-treated parietal cells are shown in fig.1. The elemental concentrations of the apical cytoplasm in both resting and stimulated states are also listed in table 1. When treated with gastrin the Ca^{2+} concentration in the apical cytoplasm surrounding the enlarged secretory canaliculi increased after 3 min, indicating that gastrin enhances an increase in cytoplasmic free Ca^{2+} concentration in the regional areas. Most of the Ca^{2+} seemed to be in a bound form because the Ca^{2+} level is too high for free Ca^{2+}. Simultaneously, there was an increase in Na^+ and Cl^- concentrations while the K^+ level decreased in the apical cytoplasm during stimulation.

3.2. *Ca^{2+} mobilization brought about by IP_3 and gastrin*

Fig.2A depicts ATP-dependent $^{45}Ca^{2+}$ uptake by the subcellular fraction (smooth-surfaced membrane fraction) obtained from sucrose density gradient centrifugation. The cholesterol (mmol)/

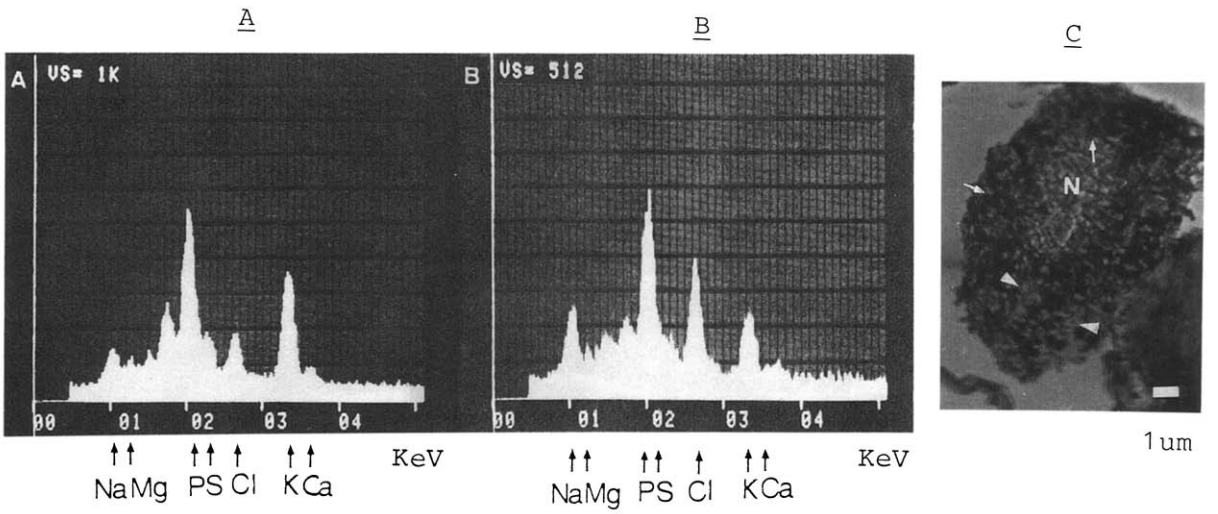

Fig.1. X-ray spectra from resting apical cytoplasm (A) and gastrin (10^{-8} M)-stimulated apical cytoplasm (B). Spectra are representative of at least 6 similar determinations. (C) Electron micrograph of a resting parietal cell from a non-fixed frozen thin section. N, nucleus; arrow, mitochondria; arrowhead, apical cytoplasm.

phospholipid (mmol) ratio of this fraction, measured by using $FeCl_3/H_2SO_4$ (cholesterol) [22] and by the procedure of Bartlett (phospholipid) [23], was 57.8% (1.156 ± 0.070; n = 9) of that measured by microsomes which contained abundant plasma membrane vesicles (2000, n = 2). The functions and characteristics of ATP-dependent Ca^{2+} uptake by vesicles obtained here were distinguishable from those by plasmalemma because of their requirement for oxalate and insensitivity to saponin [13]. The Ca^{2+} ionophore A23187 plus EGTA, but not EGTA alone, led to a rapid release of $^{45}Ca^{2+}$ accumulated in vesicles, indicating that the Ca^{2+} sequestered by ATP exists in internal store(s) and is not bound to their exterior. The ATP-dependent $^{45}Ca^{2+}$ uptake by smooth-surfaced membrane vesicles from a parietal cell [with ATP 1.27 ± 0.05 (n = 18), without ATP 0.05 ± 0.02 (n = 18) fmol/cell per 20 min, respectively] was very similar to that taken up by a single plasmalemmal permeabilized (with saponin) and mitochondrial poisoned (using antimycin and 2,4-dinitrophenol) parietal cell [with ATP 1.56 ± 0.60 (n = 3), without ATP 0.306 (n = 2) fmol/cell per 20 min, respectively; see fig.2D]. Therefore, it seems that the Ca^{2+} mobilization in permeable cells may reflect that in non-mitochondrial location(s) such as smooth-surfaced membranes. As shown in fig.2D, there was rapid uptake of $^{45}Ca^{2+}$ by permeable cells following addition of ATP, reaching a steady state at 20 min. At 20 min, IP_3 was added, leading to a 53% loss of cellular $^{45}Ca^{2+}$ content over a 1 min period [from 1.56 ± 0.60 to 0.65 ± 0.04 (n = 3) fmol/cell per min]. The Ca^{2+} release promoted by IP_3 was

Table 1

Concentration of Na, Mg, P, S, Cl, K and Ca in the apical cytoplasm of isolated parietal cells measured by electron probe X-ray microanalysis

	Apical cytoplasm	
	Resting state (A)	Stimulated state (B)
Na	34 ± 4	49 ± 8[a]
Mg	17 ± 2	29 ± 4
P	83 ± 13	96 ± 7
S	10 ± 6	27 ± 6
Cl	24 ± 6	86 ± 17[a]
K	107 ± 12	55 ± 11[a]
Ca	1.0 ± 0.3	5.2 ± 1.6[a]
n	(10)	(6)

Values are means ± SD (mmol/kg wet wt). Dry mass fraction of the apical cytoplasm was 25%. B: gastrin (10^{-8} M)-stimulated apical cytoplasm. Significant difference was calculated from each corresponding resting value ([a] $P < 0.05$) using the unpaired t-test

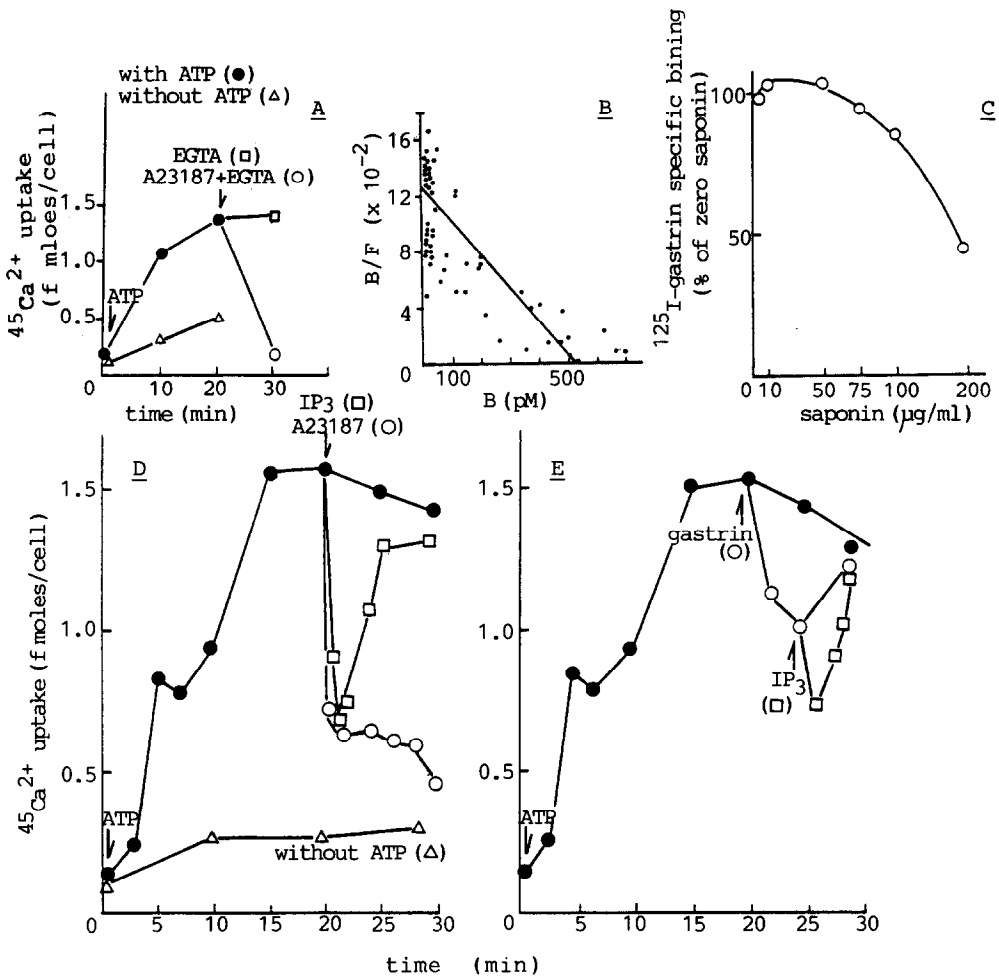

Fig.2. Ca^{2+} mobilization brought about by IP_3 and gastrin in the parietal cell. Time course of ATP-dependent $^{45}Ca^{2+}$ uptake into smooth-surfaced membrane-enriched vesicles (A). Scatchard representation (B). Effects of saponin on ^{125}I-gastrin binding to cells (C). ^{125}I-gastrin binding is expressed as the specific binding at 15 min. 100% corresponds to ^{125}I-gastrin-specific binding in the absence of saponin. $^{45}Ca^{2+}$ released by IP_3 and gastrin, accumulated by ATP in saponin-permeabilized cells (D,E). Concentrations of reagents: 1.5 mM ATP, 5 μM IP_3, 5 μg/ml A23187, 10 nM gastrin. Each datum is a representation from 4 separate experiments.

transient, since a rapid reuptake of $^{45}Ca^{2+}$ during the 14 min period after IP_3 stimulation was manifested. A23187 caused the release of $^{45}Ca^{2+}$ by 5 min after stimulation [to 0.62 ± 0.04 (n = 12) fmol/cell per 5 min] without promoting Ca^{2+} reuptake. As shown in fig.2B, there exists a gastrin receptor in the parietal cell for which the K_d (dissociation constant) amounts to 4.098 × 10^{-9} M, the number of binding sites being 79000 from a single cell [bound(B)/free(F) = $-0.000244B + 0.128000$ (n = 58), correlation coefficient for the relation (r) = 0.755 ($p < 0.001$)].

Since 90% of the ^{125}I-gastrin could bind to 75 μg/ml saponin-treated cells (10^6 cells), permeable cells might retain the ability to react to gastrin with Ca^{2+} release (fig.2C). The protein content of a parietal cell is 7.9 ng [13]. Since saponin permeabilization was accomplished at a concentration of 75 μg/7 mg protein per ml in hepatocytes [24] and 50 μg/4.5 mg protein per ml in gastric chief cells [14], about 10 μg saponin/mg protein cells per ml appears to be suitable for investigation of cell function in the permeabilized system. When gastrin was added to the incubation medium

20 min after ATP addition, there was a 25% loss of $^{45}Ca^{2+}$ followed by reuptake of Ca^{2+} to the pre-stimulation value. When IP_3 was added after gastrin, there was a further $^{45}Ca^{2+}$ release and the sum of the $^{45}Ca^{2+}$ released by gastrin plus IP_3 was almost the same as that released by IP_3 alone (fig.2E).

3.3. *Ca^{2+} mobilization brought about by TPA, OAG and gastrin*

Fig.3 shows the $[Ca^{2+}]_i$ transient measured by using aequorin. TPA, OAG and gastrin each led to an increase in $[Ca^{2+}]_i$ in both the presence and absence of medium Ca^{2+}. The magnitude of the medium Ca^{2+}-independent increase in $[Ca^{2+}]_i$ evoked by these ligands was similar to that observed with medium Ca^{2+}, suggesting that the initial peak of the aequorin signal is due to the Ca^{2+} released from the intracellular store(s). However, in the presence of medium Ca^{2+}, the TPA, OAG or gastrin-induced aequorin signal did not return to the pre-resting level and maintained a small elevation of $[Ca^{2+}]_i$. This sustained but small increase in $[Ca^{2+}]_i$ might be due to the Ca^{2+} entered from the extracellular space, since addition of the Ca^{2+} entry blocker lanthanum after peak formation immediately decreased the aequorin signal to the pre-resting level, and the medium Ca^{2+}-independent but transient increase in aequorin signal was followed by a return to the pre-resting level. The Ca^{2+} ionophore ionomycin-induced increase in $[Ca^{2+}]_i$ was almost dependent on medium Ca^{2+}.

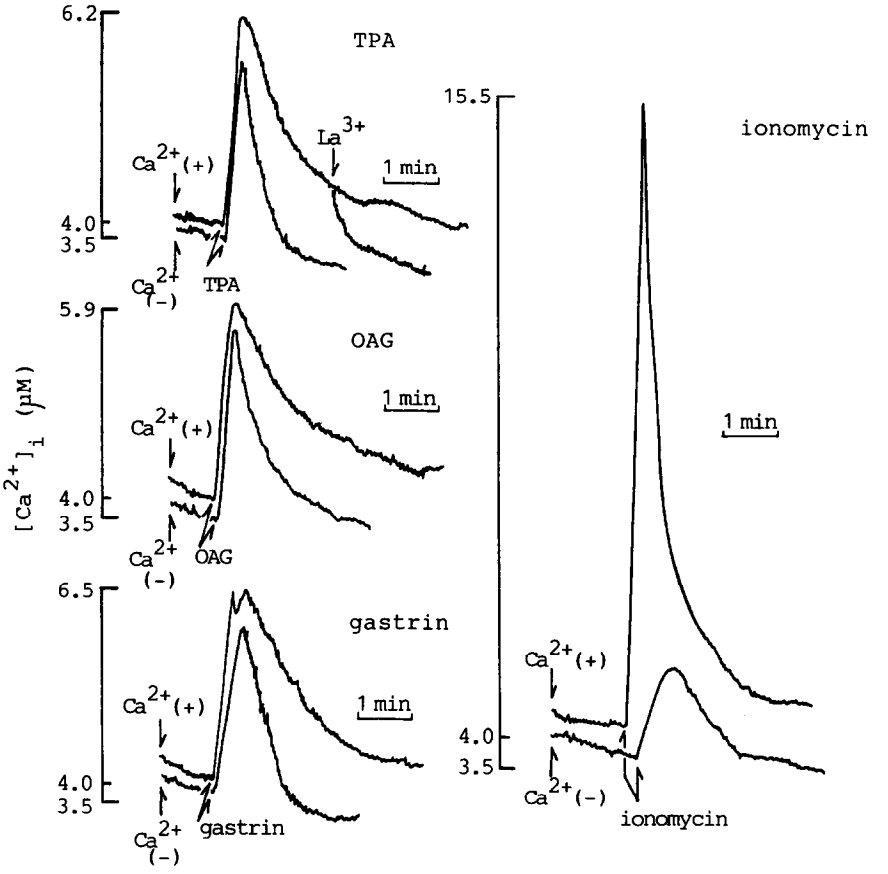

Fig.3. Cytoplasmic free Ca^{2+} concentration in response to gastrin (10 nM), TPA (100 nM), OAG (100 μM) or ionomycin (1 μM) as measured with aequorin. La^{3+}, lanthanum chloride at 100 μM. Measurements with La^{3+} required depletion of PO_4^{2-} from the medium to prevent La^{3+} precipitation. Medium Ca^{2+} (+) or (−) was 1 mM Ca^{2+} and 1 mM Ca^{2+} plus 2 mM EGTA in Mops-Tyrode's solution, respectively. The trace is representative of at least 4 similar experiments.

In the presence of medium Ca^{2+}, ionomycin caused a brisk rise to a peak and the following sharp decay of the aequorin signal, reflecting a system in which Ca^{2+} rapidly enters the cytosol, providing a rapid but high peak of level and diffusing throughout the cytosol without mediating ATP-promoted Ca^{2+} removal. This might be identical with the fact that the ionomycin-induced increase in $[Ca^{2+}]_i$ measured by fura-2 was maintained without a return to the resting level (see fig.4), since the Ca^{2+} gradient has dissipated throughout the cytoplasm [13].

To determine whether gastrin, TPA and OAG share common Ca^{2+} pathway(s), parietal cells were prestimulated with TPA (or OAG) and gastrin added after $[Ca^{2+}]_i$ had attained a value close to the steady-state level (table 2). Pre-stimulation of parietal cells with TPA (or OAG) substantially reduced the response to gastrin, and vice versa, suggesting that TPA and OAG act on the same Ca^{2+} pathway(s) as gastrin. This effect was not due to the consumption of aequorin in response to the initial stimulation, since the addition of ionomycin was still capable of eliciting an increase in $[Ca^{2+}]_i$ following prestimulation that was equal to the response to ionomycin without prestimulation.

The $[Ca^{2+}]_i$ response to gastrin monitored with fura-2 also demonstrated stimulation of Ca^{2+} mobilization from intracellular store(s) (fig.4). This increase in $[Ca^{2+}]_i$ in response to gastrin (by fura-2) was lower relative to that observed with aequorin measurement [with medium Ca^{2+}, from 205.96 ± 15.37 to 251.98 ± 37.72 nM (n = 5); without medium Ca^{2+}, 186.04 ± 23.40 to 226.55 ± 8.55 nM (n = 4)]. TPA and OAG did not produce any $[Ca^{2+}]_i$ changes at least as estimated from

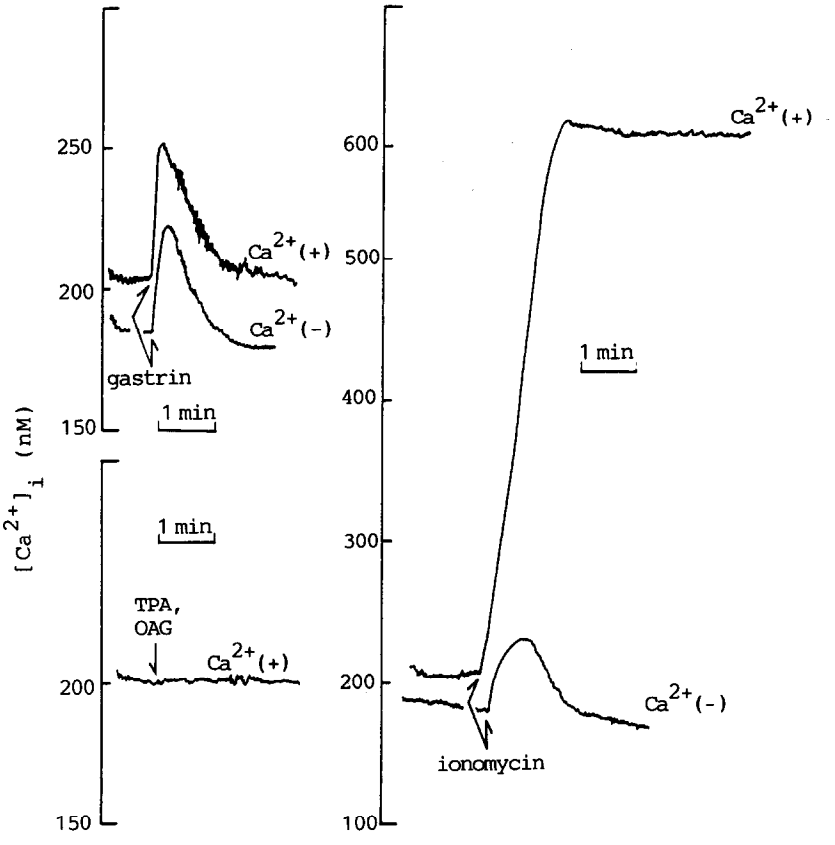

Fig.4. Cytoplasmic free Ca^{2+} concentration in response to gastrin (10 nM), TPA (100 nM), OAG (100 μM) or ionomycin (1 μM) as measured by fura-2. Medium Ca^{2+} (+) or (−) was 1 mM Ca^{2+} and 0 mM Ca^{2+} plus 1 mM EGTA in Mops-Tyrode's solution, respectively. $[Ca^{2+}]_i$ was calculated using the formula in [30]. The trace is representative of at least 4 similar experiments.

Table 2

Secretagogue-induced $[Ca^{2+}]_i$ change after prestimulation of aequorin-containing parietal cells with each ligand

Ligands	$\Delta[Ca^{2+}]_i$ increase (μM) (stimulated cells minus resting cells)			
	Control (each ligand)	After TPA	After OAG	After gastrin
TPA	2.20	0	0	0
OAG	1.90	0	0	0.18
Gastrin	2.50	0	0.25	0
Ionomycin	11.50	11.31	12.89	12.00

Concentrations of reagents: TPA, 100 nM; OAG, 100 μM; gastrin, 10 nM; ionomycin, 1 μM. Medium Ca^{2+} was 1.0 mM. Stimulation by each ligand was carried out after 1 min when the cells were firstly stimulated by either TPA, OAG or gastrin. Data represent means from 2 separate experiments

determinations with fura-2. The result suggests that fura-2 and aequorin provide information about different aspects of Ca^{2+} homeostasis during TPA (and/or OAG) stimulation. In the presence of medium Ca^{2+} ionomycin caused a large increase in $[Ca^{2+}]_i$ without decay of the fura-2 signal after peak formation [from 205.96 ± 15.37 to 634.67 nM (n = 2)].

4. DISCUSSION

Since in the gastric parietal cell an ATP-dependent and IP_3-sensitive Ca^{2+} pool connected with microfilaments is in the apical portion and gastrin induces intracellular Ca^{2+} release from the store(s) through the cytoskeletal-regulated system [3,4,12], it seems likely that the gastrin-elicited Ca^{2+} release from the store(s) is mediated by IP_3. This was further justified by the fact that the sum of the Ca^{2+} released from the store(s) by gastrin and IP_3 was constant in saponin-permeabilized cells. The constant but non-additive effect on Ca^{2+} release from the store(s) induced by the combination with gastrin and IP_3 implies that gastrin-induced Ca^{2+} release occurs at the IP_3-sensitive Ca^{2+} pool, since if the Ca^{2+} release elicited by gastrin and that mediated by IP_3 occur via different pathways, a further amount of Ca^{2+} release should be observed. Parietal cells containing aequorin showed high rates of utilization corresponding to 3–4.5 μM (assuming intracellular $[Mg^{2+}]$ to be 1 mM) as compared to an estimate of 0.2 μM using fura-2. The reason for this high value is not clear and the precise location of aequorin in parietal cells introduced by means of the dimethyl sulfoxide technique is uncertain. However, it seems unlikely that the high $[Ca^{2+}]_i$ is due to increased permeability in light of the fact that in the absence of medium Ca^{2+}, the resting $[Ca^{2+}]_i$ indicated by aequorin luminescence is of the order of micromolar and that a transient increase in $[Ca^{2+}]_i$ is detectable upon stimulation of these parietal cells with gastrin, TPA or OAG. This in turn suggests the possibility that there are Ca^{2+} gradients in the cytoplasm with inhomogeneity in $[Ca^{2+}]_i$. The diacylglycerols, therefore, induce $[Ca^{2+}]_i$ changes that can only be detected with aequorin and are too localized to be detected with fura-2. A similar phenomenon was observed in aequorin-containing platelets introduced by EGTA at low temperature [19,25]. The elevation of localized $[Ca^{2+}]_i$ brought about by TPA or OAG follows a similar pathway(s) of Ca^{2+} mobilization to that of gastrin, via intracellular Ca^{2+} release from the store(s) and the subsequent late, sustained and more localized elevation of $[Ca^{2+}]_i$ due to the Ca^{2+} entered from the extracellular space, since prestimulation of cells with TPA or OAG prevented the increase in the gastrin-elicited aequorin signal and vice versa.

The small increase in $[Ca^{2+}]_i$ in response to gastrin monitored with fura-2 is likely to be due to IP_3-mediated Ca^{2+} release from the store(s) independently of that induced by TPA (or OAG).

The Ca^{2+} shifts caused by gastrin and the involvements of IP_3 and protein kinase C in producing those shifts may contribute to production of the gastrin-induced biphasic acid secretion that comprises two components: an initial acid secretion that is independent of and a sustained response that does depend on medium Ca^{2+} [12]. Furthermore, X-ray spectra indicate that the gastrin-mediated and possibly IP_3-sensitive Ca^{2+} pool is in or near the apical portion of the gastric parietal cell, since a 5-fold increase could be detected in $[Ca^{2+}]_i$ in the restricted apical cytoplasm in individual cells during gastrin stimulation.

The source of the TPA (or OAG)-sensitive and aequorin-detectable Ca^{2+} changes has not yet been established, however, the prolonged but more localized elevation of $[Ca^{2+}]_i$ brought about by

TPA (or OAG) or gastrin is mediated by the Ca^{2+} entry from the extracellular space. It seems that the Ca^{2+} which entered from the extracellular space during a late phase plays an essential role in causing and maintaining sustained acid secretion [12]. The transient aequorin signal may reflect the rapid Ca^{2+} removal brought about by TPA (or OAG), since it enhances Ca^{2+} efflux in other types of cell [27]. This signal could result from the action of protein kinase C rather than being required for its activation. Our X-ray spectra showed that gastrin led to an increase in concentration of cytoplasmic Na^+ simultaneously with increasing Cl^- and decreasing K^+ in the cytoplasm, suggesting the possibility that the cytoplasmic pH change, such as that due to Na^+-H^+ antiport, is correlated with Ca^{2+} entry in a sustained phase, as proposed in [26]. Taken together, the Ca^{2+} cycling in gastric parietal cells appears to proceed along the following steps [3,4,12,13]. (i) IP_3-induced Ca^{2+} release from the store(s), presumably smooth-surfaced membranes in the apical portion(s); (ii) Ca^{2+} efflux from the cell by a calmodulin-regulated Ca^{2+} pump or by a Na^+-Ca^{2+} antiporter; (iii) a late, sustained and more localized Ca^{2+} entry from the extracellular space and the subsequent Ca^{2+} efflux from the cell, that may be induced by diacylglycerol; (iv) ATP-dependent Ca^{2+} re-uptake into the deleted pool(s) by the Ca^{2+} entered from the extracellular space. This process may be enhanced by hormone dissociation from the receptor [28,29], leading to termination of the cell response.

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