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A role for Ca^{2+} in mediating hormone-induced biphasic pepsinogen secretion from the chief cell determined by luminescent and fluorescent probes and X-ray microprobe

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In isolated chief cells from the guinea pig, cholecystokinin (10 nM) and a high concentration of ionomycin each caused a biphasic pattern of pepsinogen secretion. The initial fast response to cholecystokinin was not dependent on medium Ca^{2+} and was mimicked by low concentration of ionomycin (100 nM). Inositol 1,4,5-trisphosphate caused a similar fast release from permeabilized cells. The slow component of release was dependent on medium Ca^{2+}, however, and was mimicked by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (100 nM) or the diacylglycerol analogue 1-oleoyl-2-acetylglycerol (OAG) (100 μM). Ionomycin (100 nM) and TPA (and/or OAG), when applied together, reproduced the biphasic pattern of pepsinogen secretion, suggesting that the signalling pathways utilized by both types of agonist contribute to the response evoked by cholecystokinin-hormone stimulation. Both fura-2 and aequorin were used to monitor changes of intracellular Ca^{2+}. Three pathways were found to contribute to the Ca^{2+} transient. A rapid release of Ca^{2+} from intracellular store(s), a rapid Ca^{2+} entry from the extracellular space, and a more sustained Ca^{2+} entry from the extracellular space. Cholecystokinin induced a rapid increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) as estimated with fura-2 and aequorin. This rise was reduced but not abolished upon removal of extracellular Ca^{2+}, suggesting that both Ca^{2+} entry from the extracellular space and Ca^{2+} mobilization from the intracellular store(s) contribute to the initial, fast component of the Ca^{2+} transient. A second, more sustained component of the Ca^{2+} transient induced by cholecystokinin was abolished by lanthanum. TPA and OAG induced a biphasic Ca^{2+} transient that could be detected only with aequorin. The late, sustained component of this response was again abolished by lanthanum as well as by removal of extracellular Ca^{2+}. It appears that the late component of the Ca^{2+} transient is dependent on Ca^{2+} influx from the extracellular space and is too localized to be detected by fura-2. Prestimulation of cells with TPA or OAG prevented the aequorin transient caused by cholecystokinin and vice versa, suggesting that TPA, OAG and cholecystokinin activate the same pathways of Ca^{2+} entry into the cytosol from the intracellular store(s) or the extracellular

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, 1-oleoyl-2-acetylglycerol; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; fura-2 acetoxymethyl ester, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methyl-phenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester; CCK-OP, cholecystokinin octapeptide.

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space. The stimulation-sensitive Ca^{2+} pool was examined with electron probe X-ray microanalysis. It appears to be restricted to an area enriched in secretory granules or peripheral endoplasmic reticulum just beneath the apical plasma membrane and in close association with the microtubular-microfilamentous system. Ionomycin stimulation decreased the Ca level in secretory granules and reciprocally increased the cytoplasmic level of Ca. These changes were accompanied by entry of Na^+ and Cl^- from the extracellular space and by K^+ efflux from the cell. Our data suggest that the initial fast component of pepsinogen secretion is associated with a high, fast and more global rise of cytoplasmic Ca^{2+}, and that the sustained component is associated with a late, prolonged and more localized elevation of cytoplasmic Ca^{2+} that is dependent on influx of Ca^{2+} from the extracellular space.

Introduction

In mammalian gastric chief cells, there are at least three classes of receptor responding to, firstly, acetylcholine via cholinergic pathway, secondly, cholecystokinin, secretin and vasoactive intestinal polypeptide via the peptidergic pathway and thirdly, the β-adrenergic pathway [1–9]. Among others, the cholinergic and cholecystokinin receptors share a common intracellular mechanism, perhaps mediated by Ca^{2+}, thus differing from β-adrenergic and some peptidergic pathways which produce cyclic AMP [1–9]. Cellular Ca^{2+} mobilization induced by peptidergic (cholecystokinin) and cholinergic pathway may be mediated by either Ca^{2+} release from the intracellular Ca^{2+} pool(s), Ca^{2+} entry from the extracellular space, or both [10–12]. The pepsinogen secretion that is induced by these hormones is biphasic; that is, an initial but transient pepsinogen secretion followed by a sustained pepsinogen secretion [10–12]. The first response is independent of medium Ca^{2+} and the increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the acetylcholine analogue carbachol and cholecystokinin appears to be mediated by inositol trisphosphate (IP_3) [10], suggesting that the first pepsinogen secretion is mediated by IP_3-induced Ca^{2+} release from the Ca^{2+} pool(s), presumably the endoplasmic reticulum.

On the other hand, since the phorbol esters, which activate protein kinase C [13], cause sustained pepsinogen secretion, the sustained response is probably mediated by diacylglycerol [11]. The two pathways might contribute to the final response by acting synergistically [11]. Taken together, the problems have been focused on defining the following points. (a) The relationship between Ca^{2+} mobilization and other ion movements for exocytosis. (b) The precise localization of the IP_3-sensitive Ca^{2+} pool(s). (c) A role for Ca^{2+} released from the store(s) or entered from the extracellular space in producing and maintaining pepsinogen secretion. (d) The mechanism of the synergism between initial response and sustained response. In order to elucidate these problems, we employed Ca^{2+}-indicated probes (fura-2, aequorin and chlorotetracycline), isotope $^{45}Ca^{2+}$ and X-ray microprobe in intact cells, permeable cells and the subcellular fraction. The Ca^{2+}-sensitive photoprotein, aequorin, harvested from the jellyfish *Aequorea aequoreas*, has little Ca^{2+}-buffering capacity and emits blue light upon binding Ca^{2+} up to a submicromolar range, and may be of use in the identification of local elevations of $[Ca^{2+}]_i$ since the aequorin signal is dominated by the highest $[Ca^{2+}]_i$ [14]. The Ca^{2+} indicator, fura-2, which has a higher quantum yield than quin2, could detect a broad change of $[Ca^{2+}]_i$ ranging from 10 nM to 1 μM, due to its high K_d for Ca^{2+} as compared with quin2, and could be used in the identification of $[Ca^{2+}]_i$ throughout the cytoplasm [15]. The patchy fluorescence of chlorotetracycline in the cell is assumed to arise from the inner plasma membrane [16]. Electron probe X-ray microanalysis enables a quantitative and direct estimation of the elemental composition, including Ca [17].

Materials and Methods

Materials

The sources of the reagents mentioned below had been given previously [18–21]. Inositol trisphosphate, antimycin, 2,4-dinitrophenol, creatine phosphate, creatine phosphokinase, human blood

hemoglobin, saponin, cholecystokinin octapeptide, 12-*O*-tetradecanoylphorbol 13-acetate and 1-oleoyl-2-acetylglycerol were obtained from Sigma (U.S.A.). Ionomycin Ca^{2+} salt was from Calbiochem (U.S.A.). Percoll was from Pharmacia (Sweden). Fura-2 (acetoxymethyl ester type) was from Dougindo (Japan). Aequorin was purchased from Dr. Blinks (Mayo Clinic, U.S.A.). Cholecystokinin octapeptide was stored at −20 °C in 50 mM phosphate buffer (pH 7.4)/0.15 M NaCl. 10% dimethyl sulfoxide, 675 unit per ml of kallikrein inhibitor of aprotinin and 0.2% bovine serum albumin.

Methods

Preparation of isolated chief cells. Dispersed heterogeneous gastric mucosal cells from a young male guinea pig (Hartley strain, 300 g) were prepared by a previously described method [18], which was a modification from Berglindh [22]. Mucosal cells-containing $5 \cdot 10^6$ chief cells in 1.5 ml of Hanks' balanced salts solution were mixed with 7.5 ml of oxygenated 45% Percoll-Hanks' balanced salts solution and ultracentrifuged ($30\,000 \times g$, 15 min, 4 °C) [7,12,23]. Chief cells appeared near the bottom of the gradient (density, 1.062–1.076 g/ml). The pepsinogen content of the fraction enriched in chief cells was 8-fold that in parietal cells. The abundance of chief cells was almost 82%. The viability of chief cells was almost 95%. In Fig. 7 and Table I, cell separation was effected using a Beckman elutriator rotor (Beckman, U.S.A.) (flow rate, 25 ml/min; centrifugal speed, 2000 rpm). The abundance and the viability of chief cells were similar to those taken by the isopycnic centrifugation on linear density gradient of Percoll [19–21].

Pepsinogen measurement. Pepsinogen was measured by the method of Anson and Mirsky [24] where chief cells (10^6/ml) were incubated in Hanks' balanced salts solution at 37 °C over a range of times, and then were centrifuged at $10\,000 \times g$ for 30 s. The resultant pellet was sonicated for 30 s. 100 μl of either supernatant or 20-fold diluted pellet was added to 400 μl of acidic solution (320 μl of H_2O and 80 μl of 0.3 M HCl) containing 2.5% human blood hemoglobin and was incubated for 10 min at 37 °C. The reaction was stopped by adding 1 ml of 5% trichloroacetic acid. The suspension was centrifuged at $800 \times g$ for 10 min and the optical absorbance of the supernatant read at 640 nm using tyrosine as a standard. Pepsinogen secretion is expressed as a percentage of total pepsinogen activity present in the cells plus that in the medium.

Measurement of $[Ca^{2+}]_i$ by fura-2. 2 μM of fura-2 (acetoxymethyl ester) was added to a chief cell suspension (10^7 cells/10 ml) in RPMI 1640 medium (pH 7.4) and was loaded for 15 min at 37 °C. After loading, 10^6 cells were resuspended in 2 ml of Hanks' balanced salts solution in the presence (Ca^{2+}, 1.3 mM) or absence of medium Ca^{2+} (prepared by omitting $CaCl_2$ and by adding 1 mM of EGTA) in a cuvette. Fluorescence was recorded with a Hitachi 650-60 fluorescence spectrometer (Japan) with continuous stirring. The excitation and emission wavelengths were 350 and 500 nm with 10 and 20 bandwidths, respectively. $[Ca^{2+}]_i$ is calculated using the formula [15]: $[Ca^{2+}]_i = K_d \cdot (F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant of fura-2 for Ca^{2+} (224 nM). Calibration of the fura-2–Ca^{2+} signal was made by adding 4 mM of EGTA from a 200 mM stock solution in Tris base (pH 8.3), followed by 0.1% Triton X-100 (F_{min}) and 4 mM $CaCl_2$ (total Ca^{2+}, 5.3 mM) (F_{max}). The detailed method has been described previously [12].

Measurement of $[Ca^{2+}]_i$ by aequorin. Isolated chief cells ($5 \cdot 10^6$/150 μl of Hepes-Tyrode's solution (mM: NaCl, 150; KCl, 5; $MgSO_4$, 1; KH_2PO_4, 0.2; K_2HPO_4, 0.8; Hepes, 10; glucose, 5; ATP, 1; EGTA, 2 (pH 7.4)) were transferred to 10 μl aliquots containing aequorin (0.003 mg), 5 mM Hepes, 50 mM KCl and 7 mM EGTA [25]. Cells were incubated with aequorin solution for 6 min at 24 °C by the stepwise addition of 1% dimethyl sulfoxide to obtain a final concentration of 6% with continuous stirring [26], and the cell suspension was then diluted with 900 μl of Hepes-Tyrode's solution-containing 1 mM ATP and 5 mM EGTA. The cell suspension was centrifuged at $12\,000 \times g$ for 15 s and the resultant pellet was resuspended in 1 ml of Hepes-Tyrode's solution. After addition of 10 μl of 100 mM $CaCl_2$ (to obtain a final concentration of 1 mM) to 1 ml of the cell suspension ($5 \cdot 10^5$ cells) in a cuvette, the aequorin luminescence was detected by use of a Platelet Ionized Calcium Aggregometer

(Chrono-Log, U.S.A.) at 37°C with continuous stirring. $[Ca^{2+}]_i$ was calibrated by the method of Allen and Blinks [27].

Measurement of $[Ca^{2+}]_i$ by chlorotetracycline. 50 μM of chlorotetracycline suspended with Tris-saline (pH 7.4) was added to a chief cell suspension (10^7 cells/10 ml) in RPMI 1640 medium containing 10 mM Hepes and 0.2% bovine serum albumin (pH 7.4), and then cells were loaded for 30 min at 37°C. 10^6 dye-loaded cells were resuspended in 2 ml of Ca^{2+}-poor medium (Hepes-Tyrode's solution) without EGTA. 20 μl of 100 mM $CaCl_2$ (to obtain a final concentration of 1 mM) was added to a cell suspension and kept for 20 min to equilibrate cellular Ca^{2+} distributions at 24°C. Fluorescence was recorded by a Hitachi 650-60 (exitation, 400 nm; emission, 530 nm) at 24°C with continuous stirring. The fluorescent change after the stimulation is expressed in arbitrary units.

Preparation of permeable cells. Isolated chief cells (10^6/ml, 4.5 mg protein/ml) were immediately resuspended in a medium resembling the cytosol, of the following composition; 20 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 0.18 μM Ca^{2+} and 25 mM $NaHCO_3$ in oxygenated Hepes (15 mM) buffer at pH 7.2. The medium also contained 2% bovine serum albumin, 45μg/ml saponin, 10 μM antimycin, 10 μM 2,4-dinitrophenol and an ATP-regenerating system consisting of 5 mM creatine phosphate and 50 μg/ml creatine phosphokinase. After an incubation period of 15 min at 37°C, the cells were spun at $100 \times g$ for 5 min and resuspended in the same medium without saponin but with 1 mM EGTA and 0.49 mM $CaCl_2$ (180 nM Ca^{2+}). Permeable chief cells suspended with 'cytosol buffer' were employed in the measurement of the IP_3-induced pepsinogen secretion and change of $[Ca^{2+}]_i$. Methods were as described in the figure legends and elsewhere [20].

Subcellular fractionation. The submicrosomal vesicles were obtained by differential centrifugation and a subsequent sucrose density gradient as previously described [21]. An aliquot (100 μg protein) of the submicrosomes was suspended in an incubation medium consisting of 100 mM KCl, 5 mM $MgCl_2$, 1.0 μCi $^{45}Ca^{2+}$, 1.0 μM $CaCl_2$ (prepared by EGTA buffer) and 20 mM oxalate in 50 mM Tris-maleate buffer (pH 7.4) in a final volume of 900 μl. At the start of the incubation, Tris-ATP (100 μl) was added to give a final concentration of 1.5 mM. After a 20 min incubation at 37°C, $^{45}Ca^{2+}$ uptake was terminated by the addition of 1 mM EGTA, and the release of $^{45}Ca^{2+}$ from the submicrosomal vesicles induced by Ca^{2+} ionophore, IP_3 or Na^+ was observed. Separation of the isotope-containing vesicles from the incubation medium was achieved by filtration as previously described [21]. The $(Ca^{2+} + Mg^{2+})$-ATPase was determined by the method of Sanui [28] following the $^{45}Ca^{2+}$ uptake study. The submicrosomal vesicles (100 μg protein) were suspended in 900 μl of incubation medium (100 mM KCl; 4.5 mM $MgCl_2$; 0.2 mM $CaCl_2$; 0.165 mM EGTA; and 20 mM oxalate in 50 mM Tris-maleate buffer (pH 7.4)) and subsequently, 100 μl of Tris-ATP was added to give a final concentration of 1.5 mM. After incubation (30°C, 4 min), 10% trichloroacetic acid was added and the mixture was centrifuged at $3000 \times g$. The phosphates remaining in the supernatant were measured for Ca^{2+}-ATPase activity after reading the optical absorbance at 720 nm. The Ca^{2+}-stimulated ATPase is expressed as the value with Ca^{2+} (30 μM) minus that without Ca^{2+} (zero Ca^{2+}; 1 mM EGTA).

High-voltage electron microscopy. Conventionally fixed and embedded chief cells were cut at 1–3 μm thickness and examined under a Hitachi H1250 M high-voltage electron microscope (National Institute of Physiological Sciences) at an accelerating voltage of 1000 kV, taken at tilting angles of ±8°.

Electron probe X-ray microanalysis. Isolated chief cells in the resting and stimulated (10 min after the ionomycin (1 μM) stimulation, 10^6 cells/ml) states were put on copper specimen holders and rapidly frozen by pressing against the wall of a copper block which had been precooled in liquid nitrogen. 0.1–0.2 μm thick cryosections were cut on the Frozen Thin Sectioner of a Porter Blum MT-2 ultramicrotome maintained at −150°C, and the sections were mounted on Au or Ti grids and transferred to an FTS freezing-dry apparatus (FTS Systems, Stone Ridge, NY, U.S.A.). The preparations were dried at 10^{-3}–10^{-4} Torr overnight and then carbon coated. The X-ray microanalysis was done using a Hitachi H-500

electron microscope interfaced with a Kevex Si (Li) detector and 5100 multichannel analyzer. The microanalyzer was operated at 75 kV. The probe current of 10^{-9}–10^{-10} A was used and analyzed for 100 s. For the estimation of local dry mass fractions, the analysis of the frozen hydrated sections was performed. The grids with frozen sections were set in the cooling specimen holder of the electron microscope (H 5001C) in the ultramicrotome and the X-ray microanalysis was immediately carried out. The X-ray energy spectra and data processing to obtain the final concentration values were performed by an on-line computer system (Hitachi MB-MA 16003, CPU: 8088, MS-DOS). Details of the procedure have been given previously [17,29].

Results

Fig. 1 shows the mode of pepsinogen secretion induced by secretagogues. Cholecystokinin (10 nM) and Ca^{2+} ionophore ionomycin (1 μM) each caused a $10.34 \pm 2.41\%$ ($n = 5$) and $11.22 \pm 0.77\%$ ($n = 6$) secretion of pepsinogen (percentage of total) from chief cells over a 30 min period, respectively, in the presence of medium Ca^{2+} (control; $3.66 \pm 0.81\%$, $n = 8$). The cholecystokinin or ionomycin-induced pepsinogen secretion was typically biphasic inasmuch as the brisk but initial secretion of pepsinogen was followed by a cumulative but sustained response (Fig. 1A). The first response induced by cholecystokinin or ionomycin was independent of medium Ca^{2+} and was mimicked with 100 nM of ionomycin or 5 μM of IP_3 in permeabilized cells (Fig. 1B). Sustained pepsinogen secretion induced by cholecystokinin or ionomycin was dependent on medium Ca^{2+} and was reproduced by the stimulation of cells with TPA (100 nM) or OAG (100 μM). TPA and OAG each caused a lag period of pepsinogen secretion for 20 min followed by an increase rate of response that was dependent on medium Ca^{2+} (Fig. 1C). The hormone-induced biphasic pepsinogen secretion was reproduced by acting in concert with a low concentration of ionomycin (100 nM), which induced an initial response, and TPA (100 nM) (and/or OAG (100 μM)), which induced a sustained response (Fig. 1D).

Fig. 2 shows the changes in $[Ca^{2+}]_i$ stimulated with cholecystokinin, ionomycin, TPA or OAG in the presence or absence of medium Ca^{2+} as estimated with fura-2. The resting level of $[Ca^{2+}]_i$ in the presence or absence of medium Ca^{2+} was 295.91 ± 31.66 nM ($n = 15$) and 125.44 ± 8.15 nM ($n = 16$), respectively. In the presence of medium Ca^{2+}, cholecystokinin (1–100 nM) induced a rapid but transient increase in $[Ca^{2+}]_i$ in a dose-dependent manner that reached 60–77 nM final rise (cholecystokinin (100 nM); 372.76 ± 38.08 nM, $n = 11$), thereafter returning to the preresting level of $[Ca^{2+}]_i$. Ionomycin (100 nM, 1 μM, 5 μM) also caused a rapid increase of $[Ca^{2+}]_i$ in a dose-dependent manner that reached 60, 130 and 200 nM final rise, respectively (ionomycin (5 μM); 495.75 ± 88.39 nM, $n = 9$). High concentration of ionomycin (1 and 5 μM) failed to cause a decrease of the fura-2 signal after the peak formation, owing to dissipation of Ca^{2+} gradients in the cytoplasm [21]. When the cells were firstly stimulated with cholecystokinin, they failed to respond to a subsequent stimulation with the same hormone, but responded to ionomycin in both presence and absence of medium Ca^{2+}, suggesting that the subsequent receptor occupancy by the same hormone after first stimulation cannot evoke a Ca^{2+} mobilization again, and that the hormone-induced Ca^{2+} mobilization is a subset of the ionomycin-induced one. Cholecystokinin and ionomycin each caused an increase in $[Ca^{2+}]_i$ in a dose-dependent manner, even in the absence of medium Ca^{2+}. The increase in $[Ca^{2+}]_i$ in the absence of medium Ca^{2+} was slight and transient but significant, reaching 11–20 nM final rise at a variety of concentrations of cholecystokinin (1–100 nM). Ionomycin (1 μM) also increased $[Ca^{2+}]_i$, which peaked at 164.66 ± 16.81 nM ($n = 7$) in the absence of medium Ca^{2+}. TPA (100 nM) and OAG (100 μM) did not produce any $[Ca^{2+}]_i$ change, at least as estimated from determinations with fura-2.

As shown in Fig. 3, depletion or elimination of Na^+ or Cl^- from the incubation medium under constant ionic strength by choline chloride (150 mM) or $NaNO_3$ (150 mM), respectively, did not influence the increase in $[Ca^{2+}]_i$ as estimated with fura-2. In this case, fura-2 fluorescence was evaluated by a 340/380 nm ratio as previously described [15] and Fig. 2 (legend).

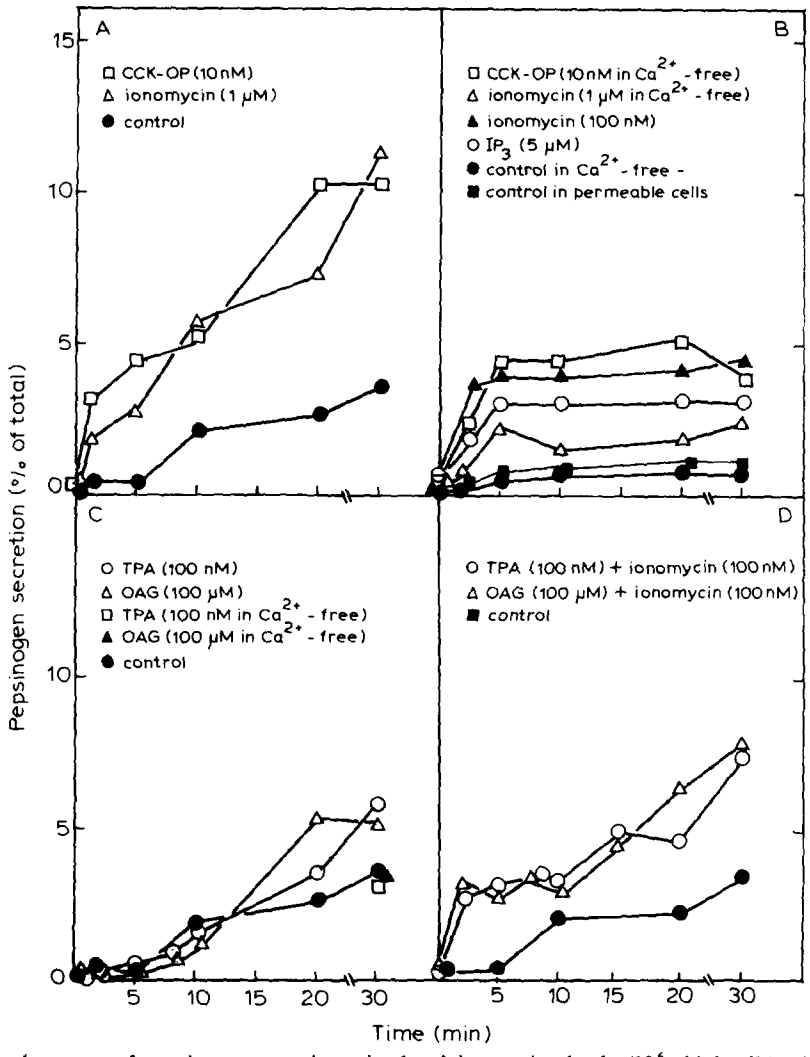

Fig. 1. Time-course and pattern of pepsinogen secretion stimulated in gastric glands (10^6 chief cells) with secretagogues. The concentration of medium Ca^{2+} was prepared by adding 1.3 mM Ca^{2+} or zero Ca^{2+} plus 1 mM EGTA (Ca^{2+}-free) in Hanks' balanced salts solution. There was no leak of pepsinogen from 45 μg/ml saponin-treated chief cells (panel B), and IP_3 was responsible for releasing Ca^{2+} from the store(s) under the same circumstances (see Ref. 12). Lactate dehydrogenase (LDH) release from intact chief cells over a 30 min period was: control, 6.33%; CCK-OP (10 nM), 7.28%; ionomycin (100 nM); 7.11%, ionomycin (1 μM), 8.20% (percentage of total, $n = 2$, respectively). LDH activity was measured by the Sigma LDH assay kit, which is a spectrophotometric assay using pyruvate and NADH as substrates [40], running in parallel with the pepsinogen measurement. There was no significant leakage of LDH after the stimulation of cells with ligands. The data represent the means from 2–7 separate experiments.

Fig. 4 shows the changes in $[Ca^{2+}]_i$ after the cell stimulation with secretagogues as measured by aequorin. The aequorin luminescence in the resting state indicated an apparent $[Ca^{2+}]_i$ of 7.1 μM in the presence of medium Ca^{2+} (1 mM) and 4.5 μM in the its absence (1 mM Ca^{2+} and 2 mM EGTA), respectively. The reason for this high value of $[Ca^{2+}]_i$ is not clear and the precise location of aequorin in chief cells introduced by dimethylsulfoxide is not certain. However, it does not necessarily follow that the high $[Ca^{2+}]_i$ observed in the aequorin-loaded cells is due to an increased permeability in light of the fact that, even in the absence of medium Ca^{2+}, the resting level of $[Ca^{2+}]_i$ indicated by aequorin luminescence is 3.5 μM for chief cells and that a

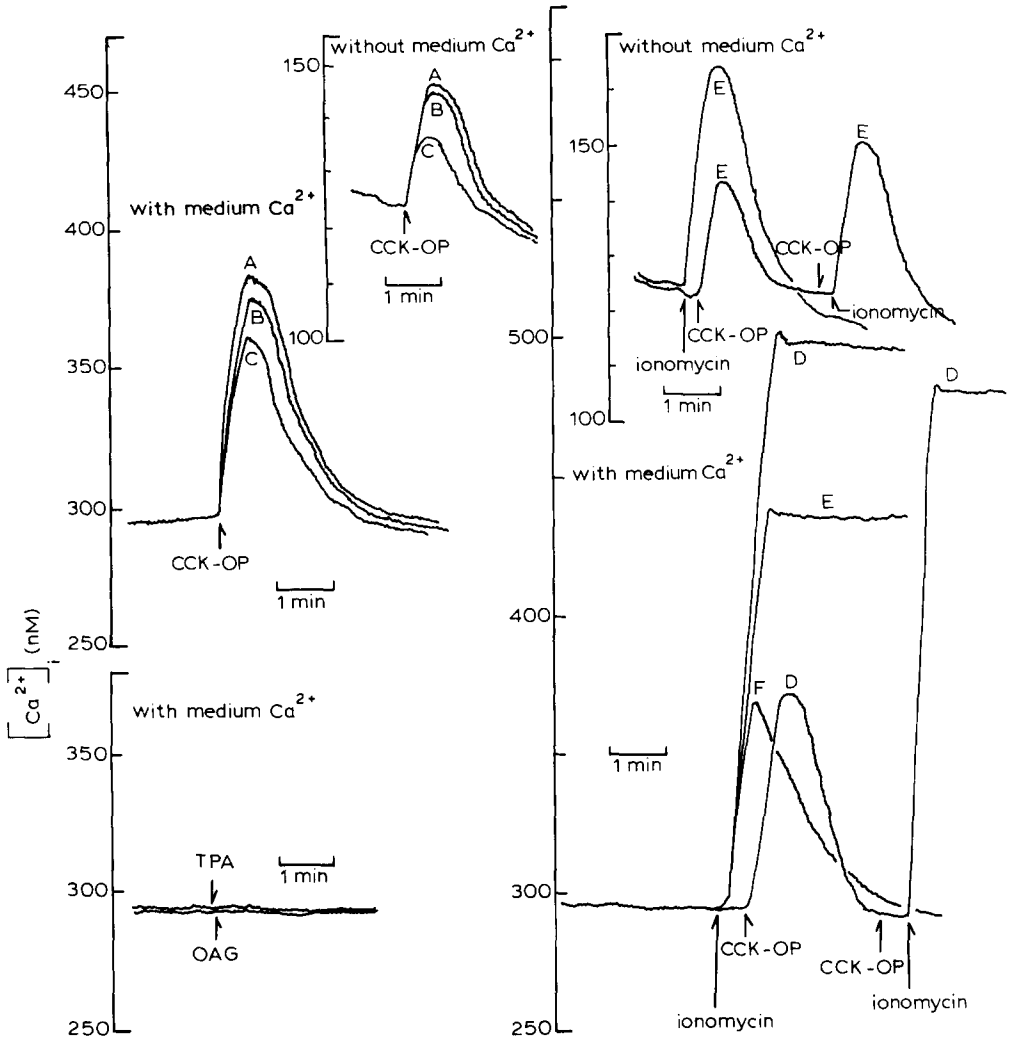

Fig. 2. Cytosolic free Ca^{2+} concentrations and responses to secretagogues measured by fura-2 (acetoxymethyl ester) in the presence or absence of medium Ca^{2+} of isolated chief cells. The concentrations of reagents tested were: CCK-OP: (A) 100 nM; (B) (including D and E), 10 nM; (C) 1 nM; ionomycin: (D) 5 μM; (E) 1 μM; (F) 100 nM; TPA, 100 nM; OAG: 100 μM. The concentration of medium Ca^{2+} was prepared by adding 1.3 mM Ca^{2+} (with medium Ca^{2+}) or zero Ca^{2+} plus 1 mM EGTA (without medium Ca^{2+}) in Hanks' balanced salts solution. EGTA was added 1 min before the secretagogue stimulation. The data represent the means from three separate experiments (8–10 determinations). If the calibration was done by fluorescence ratio (excitation, 340 nm/380 nm) [15], $[Ca^{2+}]_i$ in the resting state was 260.2 nM ($n = 2$), and CCK-OP (100 nM) and ionomycin (5 μM) each caused a 118 nM ($n = 2$) and 447 nM ($n = 2$) final rise of $[Ca^{2+}]_i$, respectively, in the presence of medium Ca^{2+}. The resting level of $[Ca^{2+}]_i$ of the aequorin-containing chief cells monitored by fura-2 was 296.0 ± 32.0 nM ($n = 3$).

transient increase in $[Ca^{2+}]_i$ is detectable upon incubation of these chief cells with cholecystokinin, TPA and OAG. In addition, the resting level of $[Ca^{2+}]_i$ of the aequorin-containing chief cells monitored by fura-2 (296.0 ± 32.0 nM, $n = 3$) was not significantly different from that of the aequorin-free cells (295.9 ± 31.7 nM, $n = 15$). The results suggest that there is an inhomogeneity of $[Ca^{2+}]_i$ in the cytoplasm. Aequorin-containing chief cells did not perturb the cholecystokinin-induced increase in $[Ca^{2+}]_i$ (monitored with fura-2) and in pepsinogen secretion (not shown). A high $[Ca^{2+}]_i$ might account for the dissociation constant in Ca^{2+}-binding protein, calmodulin (K_d =

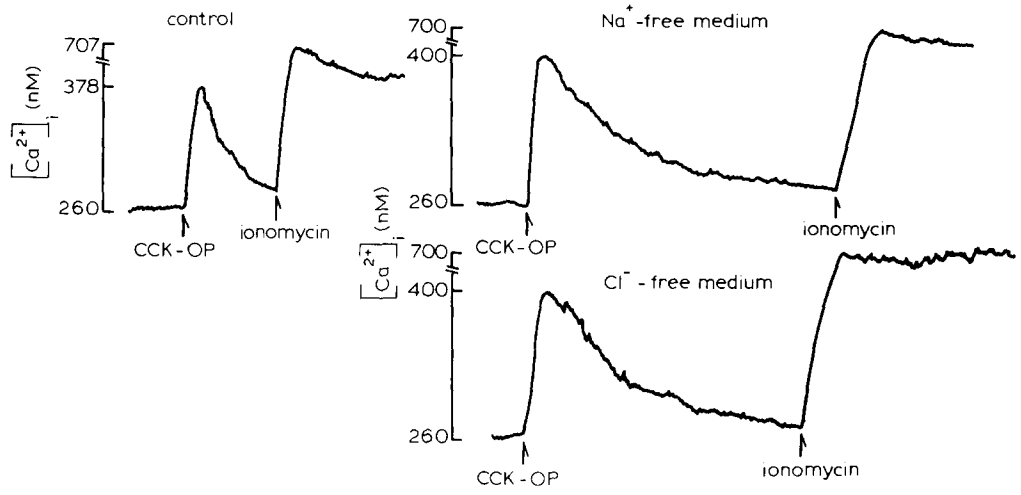

Fig. 3. Cytosolic free Ca^{2+} concentrations and responses to secretagogues measured by fura-2 (acetoxymethyl ester) in the absence of medium Na^+ and Cl^-. The concentrations of reagent tested were similar to those in Fig. 2. Na^+ or Cl^- was substituted with 150 mM choline chloride and 150 mM $NaNO_3$, respectively. The fura-2 fluorescence was read on a CAF-100 spectrophotometer (Japan Spectroscopic Company, Ltd. Japan) as described in Refs. 41, 42. The trace is representative of at least two similar experiments.

2.5 μM) [30] or for Ca^{2+}-stimulated ATPase in the submicrosomes of the chief cell, which requires a micromolar range of Ca^{2+} for its activation. Cholecystokinin (10 nM) led to an increase in $[Ca^{2+}]_i$ in the presence or absence of medium Ca^{2+} that reached 4.1 μM final rise and 2.1 μM final rise, respectively, indicating that the cholecystokinin-induced increase of $[Ca^{2+}]_i$ monitored with aequorin consists of equal amounts of Ca^{2+} release from the store(s) and of Ca^{2+} entry from the extracellular space. Though TPA or OAG failed to increase $[Ca^{2+}]_i$ as measured by fura-2, it could bring about the increase in $[Ca^{2+}]_i$ when the measurement was assessed by aequorin. The initial increase in $[Ca^{2+}]_i$ in the presence or absence of medium Ca^{2+} (evoked by TPA or OAG) was almost equal, thus suggesting that TPA or OAG leads to local Ca^{2+} elevation without diffusing Ca^{2+} throughout the cytoplasm and that the source of Ca^{2+} for local Ca^{2+} elevation in the early phase is located in intracellular space(s) of the chief cell. In the presence of medium Ca^{2+}, the aequorin signal evoked by TPA, OAG or cholecystokinin, however, remained elevated $[Ca^{2+}]_i$ and did not return to the pre-resting level. This prolonged increase in $[Ca^{2+}]_i$ may be due to the Ca^{2+} entered from the extracellular space, since addition of the Ca^{2+} entry blocker lanthanum (100 μM) in a PO_4^{2-}-free medium after peak formation decreased the aequorin signal towards the pre-resting level and a medium-Ca^{2+}-independent but transient increase in aequorin signal was followed by returning to the pre-resting level without forming a signal shoulder after the peak formation. Thus, isolated chief cells responded with a biphasic $[Ca^{2+}]_i$ change when challenged with cholecystokinin, TPA or OAG. These data are consistent with the existence of two components of $[Ca^{2+}]_i$ change: a rapid mobilization of intracellular Ca^{2+} store(s) and a more prolonged entry of extracellular Ca^{2+} too localized to be detected by fura-2.

To determine whether cholecystokinin, TPA and OAG share a common Ca^{2+} metabolic pathway, chief cells were prestimulated with TPA (or OAG) and cholecystokinin added after $[Ca^{2+}]_i$ had reached almost the steady-state level (Fig. 5). Prestimulation of chief cells with TPA (or OAG) substantially reduced the response to cholecystokinin. In the reverse experiment, prestimulation with cholecystokinin reduced the response to TPA or OAG. These results suggest that TPA and OAG act on the same Ca^{2+} pathway as cholecystokinin. This effect was not due to the consumption of aequorin in response to the initial stimulation, since the addition of ionomycin was still

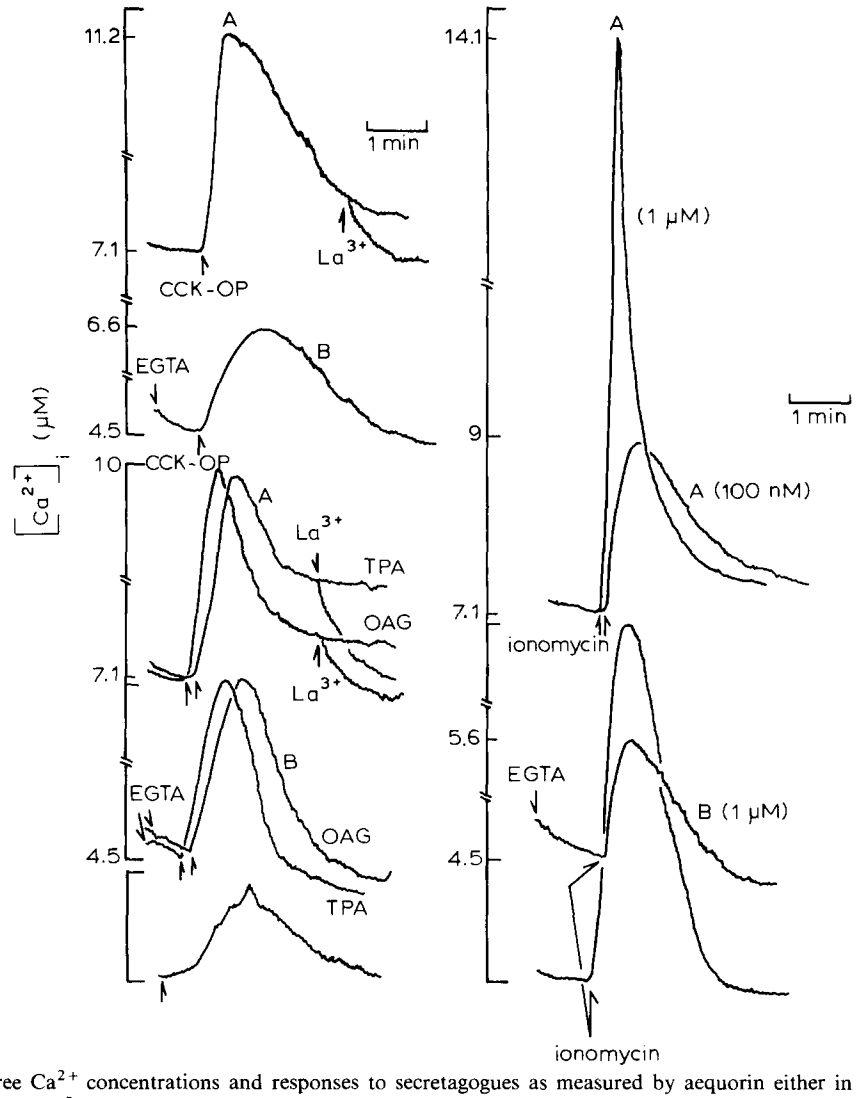

Fig. 4. Cytosolic free Ca^{2+} concentrations and responses to secretagogues as measured by aequorin either in the presence (A) or absence (B) of medium Ca^{2+}. The concentrations of reagents tested were: CCK-OP, 10 nM; ionomycin, 100 nM and 1 μM; TPA, 100 nM; OAG, 100 μM. EGTA (2 mM) was added 1 min before the secretagogue stimulation. The data are representative of a typical experiment that was repeated seven times.

capable of eliciting an increase in $[Ca^{2+}]_i$ following prestimulation that was equal to the response to ionomycin without prestimulation.

Since prolonged removal of extracellular Ca^{2+} by EGTA may alter the amount of Ca^{2+} in the pool(s), it has not been evaluated that the medium Ca^{2+}-independent increase in $[Ca^{2+}]_i$ (as measured by fura-2 and aequorin) is due to uncontaminated Ca^{2+} release from the store(s) as used by EGTA. The trivalent cation, lanthanum ($LaCl_3$), can displace Ca^{2+} in the plasma membrane and block Ca^{2+} entry across the membrane without itself entering the cells [32]. Therefore, the presence of lanthanum in the incubation medium allows the observation of only Ca^{2+} release from the store(s). Fig. 6A shows the effect of lanthanum on secretagogue-induced increase in $[Ca^{2+}]_i$ as measured by fura-2. Addition of lanthanum (100 μM) to the incubation medium prevented the cholecystokinin-induced Ca^{2+} entry from the extracellular space without inhibiting Ca^{2+} release from the store(s). Similar observation was found

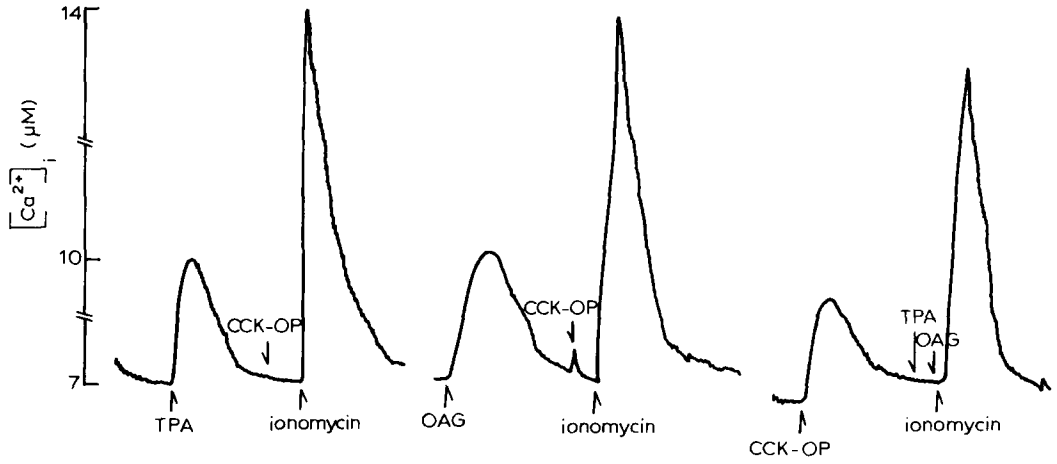

Fig. 5. Cytosolic free Ca^{2+} concentrations in response to secretagogues as measured by aequorin luminescence. The concentrations of reagents tested were similar to Fig. 4. The trace is a representative of at least five similar experiments.

in aequorin measurement (Fig. 6B). The results indicate that the increase in $[Ca^{2+}]_i$ evoked by cholecystokinin, which is independent of medium Ca^{2+}, is caused by the release of intracellular Ca^{2+} from the store(s). On the other hand, the ionomycin-induced signal was partially inhibited by lanthanum when the measurement was assessed by either fura-2 or aequorin, as shown in Fig. 6A and B, respectively. As shown in Fig. 6C, in the presence of medium Ca^{2+} the signal detected with chlorotetracycline declined upon ionomycin (1 μM) stimulation but not upon cholecystokinin stimulation. Lanthanum did not affect the ionomycin-induced decline of the signal. The patchy fluorescence of chlorotetracycline is assumed to arise from inner plasma membrane because of its clearly peripheral location [16]. It seems, therefore, that the ionomycin-induced Ca^{2+} release from the store(s) is partially derived from inner-membrane-bound Ca^{2+} in a lanthanum-insensitive manner. Ionomycin is lanthanum-sensitive only when carrying Ca^{2+} into the cell and not when releasing inner-membrane-bound or stored Ca^{2+}. There was a slight but transient increase of fluorescence of chlorotetracycline after addition of lanthanum. This may be due to the chlorotetracycline-lanthanum complex on the outer-surface membrane of the cells. On the other hand, the addition of 2 mM of EGTA caused a very rapid decline of the chlorotetracycline signal. The rapid fall of the signal brought about by EGTA might be caused by the Ca^{2+} movements from inner-membrane to extracellular space and/or outer-membrane because of different gradients of Ca^{2+} from inside to outside the cells. The rapid decrease of $[Ca^{2+}]_i$ in the resting state by an acute removal of medium Ca^{2+} with EGTA as detected with fura-2 (see Fig. 2) and aequorin (see Fig. 4) might be due to the transition of inner-membrane-bound Ca^{2+} from inside to outside the cells. It seems unlikely that the decrease of $[Ca^{2+}]_i$ in the resting state brought about by EGTA is due to outer-surface-bound fura-2 or aequorin, since the addition of 4 mM of EGTA for calibration (before Triton X-100) did not decrease the fluorescence of fura-2, and the signal of outer-surface-bound aequorin disappeared when 1 mM of Ca^{2+} was added to the incubation medium, owing to the aequorin-Ca^{2+} complex in the extracellular space. Previous data (see, Fig. 1B) indicated that the basal pepsinogen secretion was reduced after incubation of chief cells with EGTA for 10–30 min, suggesting that prolonged exposure of cells to EGTA decreases the amounts of Ca^{2+} in the store(s). Therefore, it has not been substantiated that the secretagogue-induced sustained pepsinogen secretion is dependent on medium Ca^{2+} if that is the case. However, as shown in Fig. 5D, the addition of lanthanum with cholecystokinin inhibited the sustained pepsinogen secretion, without inhibiting the initial response [12], over a range of lanthanum concentrations. Furthermore, lantha-

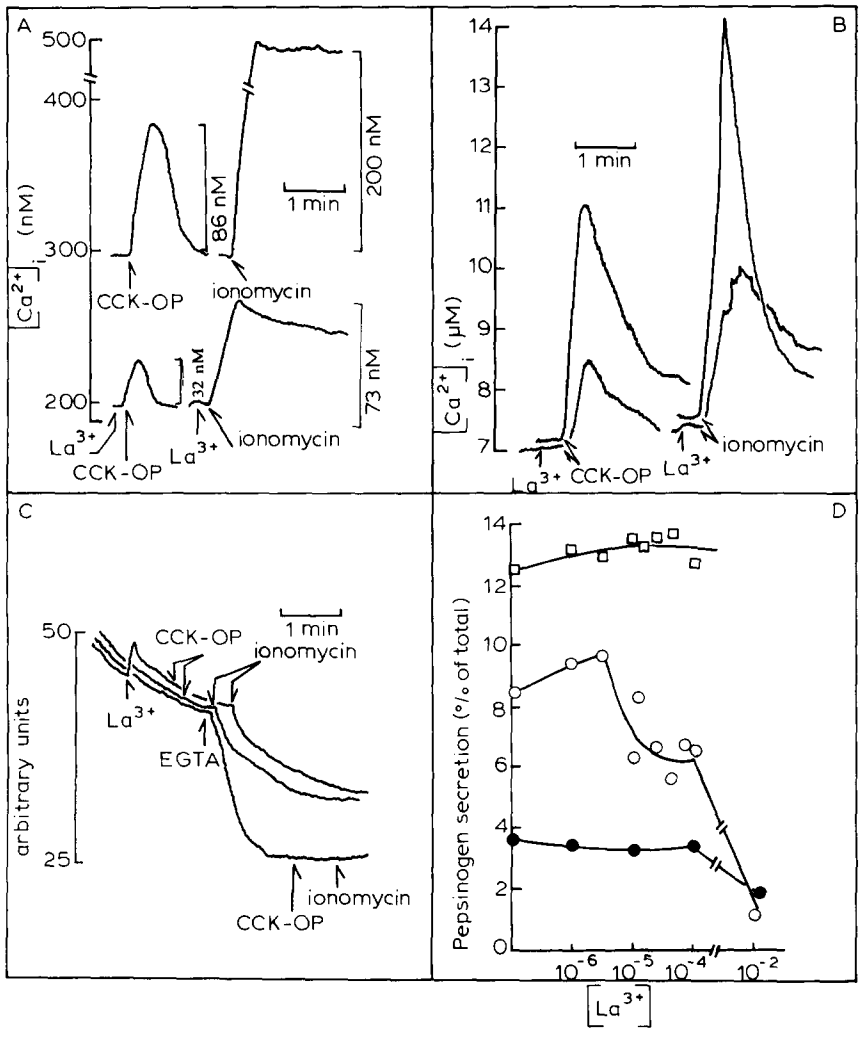

Fig. 6. Effects of lanthanum on secretagogue-induced cytosolic free Ca^{2+} concentrations ((A) fura-2; (B) aequorin; (C) chlorotetracycline) and sustained pepsinogen secretion (D) of isolated chief cells that were dependent on medium Ca^{2+}. The concentrations of reagents tested were: CCK-OP, 10 nM; ionomycin, 1 μM; lanthanum (La^{3+}), 100 μM (except (D)); EGTA, 2 mM. In panel (D) the cell suspension was incubated for 30 min with CCK-OP (and/or ionomycin) plus a variety of concentrations of lanthanum. EGTA or lanthanum was added before the secretagogue stimulation except for panel (D). The data represent means from three separate experiments. ●, Control; ○, CCK-OP; □, ionomycin.

num addition (100 μM) after initial pepsinogen secretion evoked by cholecystokinin failed to cause any subsequent sustained pepsinogen secretion. Therefore, the Ca^{2+} entry from the extracellular space, as a trigger for causing sustained pepsinogen secretion, may be evoked by an initial phase followed by a sustained phase after cell stimulation. The ionomycin (1 μM)-induced sustained response was not prevented by the addition of lanthanum over a range of concentrations. This may relate to the release of inner-membrane-bound Ca^{2+} evoked by ionomycin, which is insensitive to lanthanum.

To determine whether the IP_3-sensitive Ca^{2+} pool has an ATP-promoted Ca^{2+} removal system, the characteristics of the ATP-removal system in the subcellular fractions have been investigated. Table I shows the net and ATP-dependent Ca^{2+} uptake and the Ca^{2+}-stimulated ATPase activity in the submicrosomal vesicles from chief cells

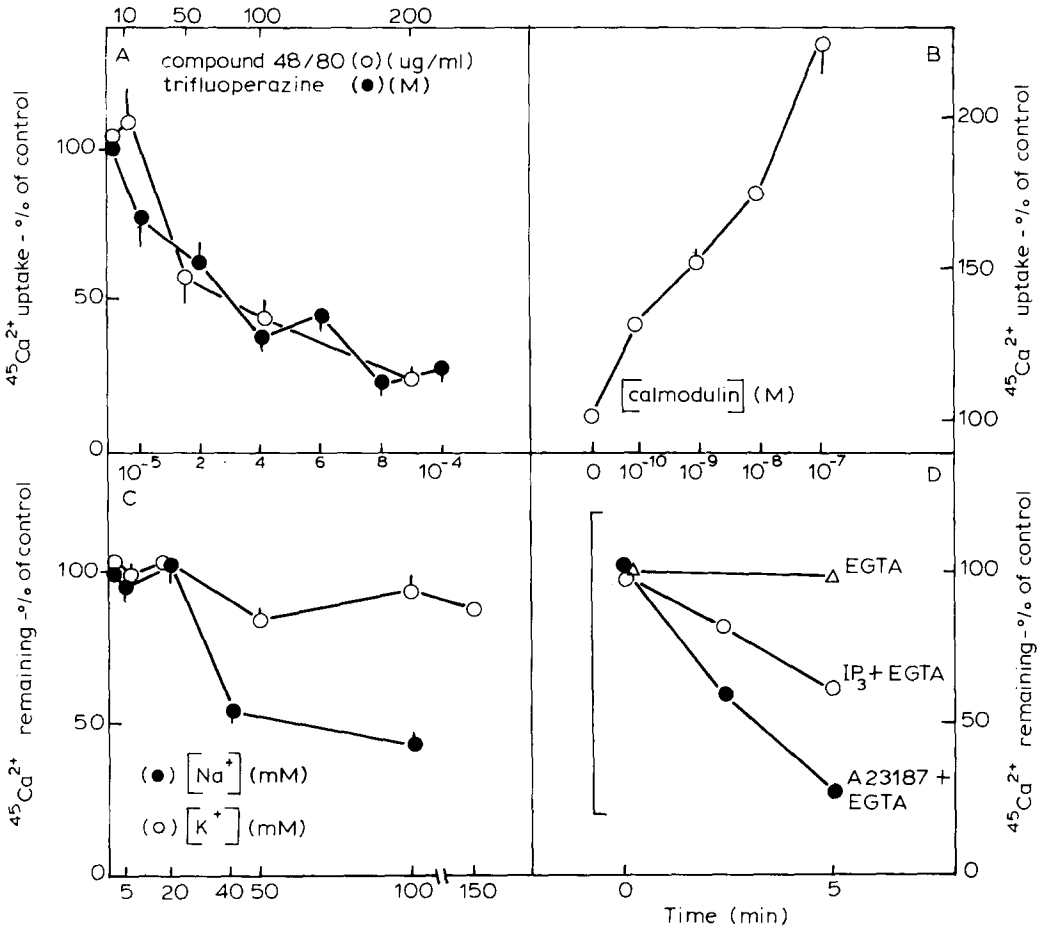

Fig. 7. The features of Ca^{2+} removing and releasing mechanism of the submicrosomal fraction from isolated chief cells. 100% corresponds to ATP-dependent $^{45}Ca^{2+}$ uptake into the post-microsomes at 20 min. ^{45}Ca taken up into the sub-microsomes by 1 mM ATP was 93.71 ± 6.07 nmol/mg protein per 20 min ($n = 8$), (without ATP 45.70 ± 4.60 nmol/mg protein per 20 min). The concentrations of reagents tested were as follows: EGTA, 1 mM; IP_3, 5 μM; A23187, 5 μg/ml. Panel D is the representation of $^{45}Ca^{2+}$ release brought about by extravesicular Na^+ after 5 min when the ATP-dependent $^{45}Ca^{2+}$ uptake was stopped by EGTA. Each point and vertical bar represents the mean $\pm$ S.E. from four separate experiments.

which were obtained by a differential centrifugation and a subsequent sucrose density gradient. The ratio of net Ca^{2+} sequestered to ATP utilized was 1.931. About 30 μM of medium Ca^{2+}, which was prepared by EGTA buffer (0.2 mM Ca^{2+} and 0.165 mM EGTA), appeared to be an appropriate concentration for the maximal Ca^{2+} uptake and also for the activation of Ca^{2+}-stimulated ATPase. The submicrosomal vesicles obtained in this study had endoplasmic reticulum, secretory granules and Golgi components rather than plasma membrane as a contamination, since the ratio of cholesterol (mmol)/phospholipid (mmol) of submicrosomes (1.385 ± 0.075, $n = 6$) was lower than that of microsomes (2, $n = 2$) (see Ref. 12). The functions and characteristics of ATP-dependent $^{45}Ca^{2+}$ uptake by submicrosomes differed from those of plasma membrane because of their requirement for oxalate and insensitivity to saponin for causing Ca^{2+} uptake (see Ref. 12). As shown in Fig. 7A, trifluoperazine and compound 48/80 each caused a dose-dependent inhibition of ATP-dependent $^{45}Ca^{2+}$ uptake with an IC_{50} (mean inhibitory concentration) of 25 μM and 80 μg/ml, respectively. The result suggests that the ATP-dependent Ca^{2+} removal system in the submicrosomal fraction is

TABLE I

A COMPARISON OF ATP-DEPENDENT NET Ca^{2+} UPTAKE AND Ca^{2+}-STIMULATED ATPase IN THE SUBMICROSOMES FROM ISOLATED CHIEF CELLS

The ATP-dependent net Ca^{2+} uptake was obtained by subtracting the net Ca^{2+} uptake when the incubation was done without ATP. The data represent the mean ± S.E. of the number of samples in parenthesis from three separate experiments.

	Net Ca^{2+} uptake (nmol/mg protein per min)	ATPase activity (nmol P_i/mg protein per min)
Ca^{2+} ($3 \cdot 10^{-5}$ M)	54.91 ± 12.80 (5)	57.72 ± 12.49 (7)
Ca^{2+}-free		29.29 ± 10.96 (7)
ΔCa^{2+}		28.44 ± 10.00 (7)
	net Ca^{2+} uptake/Ca^{2+}-stimulated ATPase = 1.931	

regulated by calmodulin. To explore the involvement of calmodulin on ATP-promoted Ca^{2+} removal in post-microsomes, the vesicles were exposed to 1 mM of EDTA (to remove endogenous calmodulin) and the effect of exogenous calmodulin was examined. The $^{45}Ca^{2+}$ taken up by submicrosomes was reduced by 60% (from 2.40 ± 0.15, $n = 8$, to 0.96 (100%), $n = 2$, fmol/cell per 20 min) after the treatment of submicrosomes with EDTA for 10 min at 30 °C. The addition of exogenous calmodulin to the submicrosomes caused an increase of $^{45}Ca^{2+}$ uptake in a dose-dependent manner, exceeding the control by 2.27-fold (from 0.96, $n = 2$, to 2.17 ± 0.02, $n = 4$, fmol/cell per 20 min) at a concentration of 100 nM (calmodulin) (Fig. 7B). Increasing concentrations of extravesicular Na^+ (40–100 mM), but not that of K^+, caused a release of ATP-accumulated $^{45}Ca^{2+}$ from submicrosomes within 5 min (Fig. 7C). A release of $^{45}Ca^{2+}$ was also observed by the stimulation with Ca^{2+} ionophore A23187 (5 μg/ml), thus indicating that $^{45}Ca^{2+}$ taken up into the vesicles was sequestered into their internal space and not superficially bound to their exterior. Similarly, IP_3 (5 μM) caused a rapid release of ATP-accumulated $^{45}Ca^{2+}$ from vesicles, the amount of which was about half of that evoked by A23187 and was almost equal to that evoked by 40 mM Na^+, thus indicating that the submicrosomal vesicles that comprise endoplasmic reticulum, secretory granules and Golgi components are the source of the IP_3-sensitive Ca^{2+} pool (Fig. 7D), and that the IP_3-sensitive and ATP-dependent Ca^{2+} pool which releases and takes up Ca^{2+}, respectively, is the same or similar.

Identification and localization of the source of intracellular Ca^{2+} pool(s) were determined by an

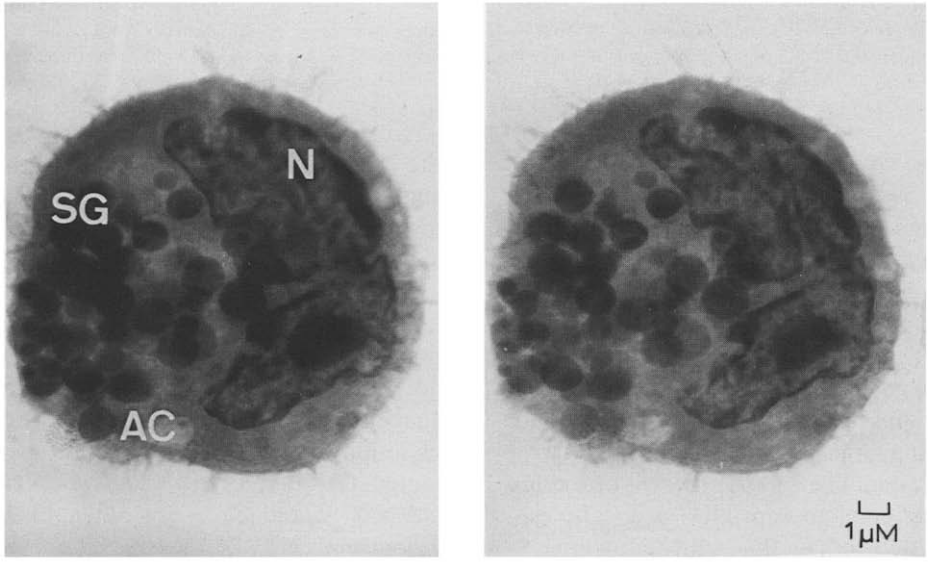

Fig. 8. Stereo-image of high-voltage electron micrograph of the chief cell. The electron micrograph is of a chief cell stimulated with ionomycin (1 μM) for 10 min at 37 °C. Tilting angle, ±8°. SG, secretory granules; N, nucleus; AC, apical cytoplasm. (×6700).

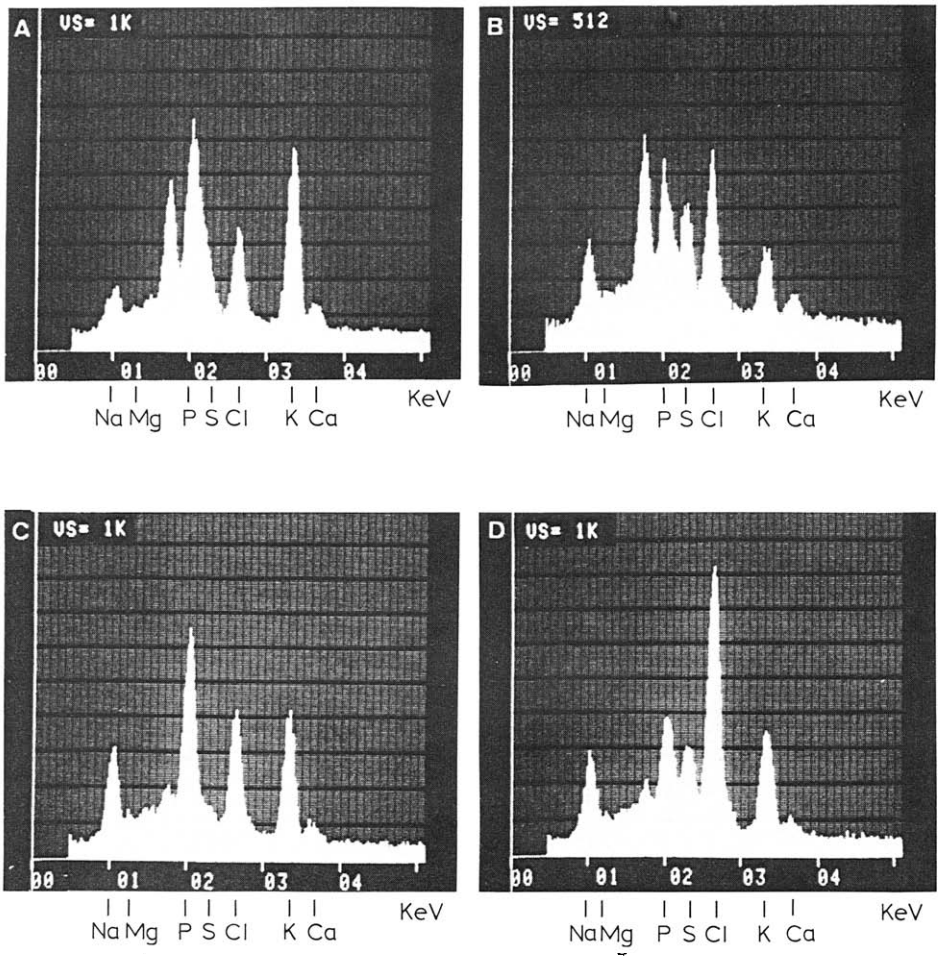

Fig. 9. Representative X-ray spectra from resting cytoplasm (A), resting granules (B), stimulated cytoplasm (C) and stimulated granules (D) of the isolated chief cell. The spectra are representative of at least three similar experiments.

electron probe X-ray microanalyzer. A typical X-ray spectrum over the cytoplasm of resting chief cell showed high peaks for P and K and low peaks for Na, S and Cl, which represents a typical cytoplasmic ion distribution (Fig. 9 and Table II). The average elemental concentrations in both the cytoplasm and secretory granules are shown in Table II. The stereo image of the high voltage electron micrograph of chief cells stimulated with ionomycin is shown in Fig. 8. The abundant secretory granules extending into the apical portion are clearly and stereomicroscopically seen. The isolated chief cells used in this study appeared to maintain their polarity as evidenced by the clustering of zymogen granules at what was presumably the apical portion of the cell. Ionomycin was employed as a secretagogue to explore the ion distributions after the cell stimulation for the following reason. Since a physiological ligand, such as cholecystokinin and IP_3, causes a rapid reuptake of released Ca^{2+} into the store(s) [12], the exact distribution of Ca^{2+} in a running time cannot be determined by electron probe X-ray microanalysis. On the other hand, a high concentration of ionomycin prevents the removal of ATP-promoted Ca^{2+}, since no Ca^{2+} gradient is formed (see Fig. 2 and Ref. 12). After stimulation with ionomycin (1 μM) for 10 min, there was an increase in intracellular Na^+, Cl^- and Ca concentrations, while the K^+ concentration decreased in the

TABLE II

CONCENTRATIONS OF Na, Mg, P, S, Cl, K AND Ca IN THE CYTOPLASM AND SECRETORY GRANULE OF ISOLATED CHIEF CELLS MEASURED BY ELECTRON PROBE X-RAY MICROANALYSIS

Mean ± S.D., mmol/kg wet weight. Dry mass fractions of the cytoplasm and secretory granules were 25% and 35%, respectively. Significant difference was calculated from corresponding resting value (a–g) (* $P < 0.05$) using the unpaired *t*-test.

	Resting cytoplasm	Resting secretory granule
Na	14.2 ± 2.9 [a]	23.2 ± 7.9 [b]
Mg	25.7 ± 0.7	34.8 ± 2.5
P	111.9 ± 10.7	61.6 ± 16.5
S	17.2 ± 1.1	38.0 ± 7.7
Cl	29.3 ± 4.1 [c]	55.3 ± 5.0 [d]
K	114.4 ± 4.8 [e]	57.2 ± 5.3
Ca	2.5 ± 0.5 [f]	7.7 ± 1.0 [g]
n	(10)	(10)
	Stimulated cytoplasm (1 μM ionomycin)	Stimulated secretory granule (1 μM ionomycin)
Na	42.4 ± 4.1 [a]*	55.4 ± 4.4 [b]*
Mg	38.5 ± 2.7	43.7 ± 0.3
P	129.6 ± 27.2	76.7 ± 5.3
S	22.9 ± 12.5	36.4 ± 12.7
Cl	67.0 ± 11.4 [c]*	118.9 ± 15.1 [d]*
K	86.4 ± 7.7 [e]*	67.1 ± 7.2
Ca	4.5 ± 1.4 [f]*	5.8 ± 0.4 [g]*
n	(10)	(10)

cytoplasm. The Na^+ and Cl^- concentrations increased, while the Ca concentration decreased in the secretory granules during ionomycin stimulation.

Discussion

Pepsinogen secretion

Cholecystokinin and a high concentration of ionomycin (1 μM) each caused a biphasic pepsinogen secretion in the presence of medium Ca^{2+}. An initial but transient response reaching a peak in 5 min was followed by a sustained response reaching a peak in 30 min. The former was reproduced by either cholecystokinin and a high concentration of ionomycin in the absence of medium Ca^{2+} including EGTA, a low concentration of ionomycin (100 nM) or IP_3 under the saponin-permeabilized circumstances. Permeabilization of 10^6 chief cells (4.5 mg protein) per ml with 45 μg of saponin (20 min, 37 °C) kept the ability to react to IP_3 with transient Ca^{2+} release from the store(s) and to obviate the pepsinogen leak from the cells [12]. The latter required medium Ca^{2+}, and was reproduced by the protein kinase C activator, phorbol ester (TPA) or exogenous diacylglycerol (OAG) on a small scale.

The TPA- or OAG-induced pepsinogen secretion was dependent on medium Ca^{2+}. The two pathways might contribute to the final response by acting synergistically, since using the combination of 100 nM ionomycin and TPA (and/or OAG), the cholecystokinin-elicited biphasic pattern of pepsinogen secretion could be mimicked. Synergism between IP_3 and TPA (and/or OAG) in exerting pepsinogen secretion has not been manifested, since TPA and OAG could not react in permeable cells, possibly owing to increased permeability of the plasma membrane in which protein kinase C might be located (not shown). The results suggest that the initial but transient pepsinogen secretion during cholecystokinin stimulation is mediated by IP_3 and the following response is mediated by diacylglycerol in chief cells hitherto described [10–12].

Initial pepsinogen secretion and Ca^{2+} mobilization

Preceding the cholecystokinin-induced initial but transient pepsinogen secretion, cholecystokinin stimulation caused a rapid but transient increase in $[Ca^{2+}]_i$ throughout the cytosol, even in the absence of medium Ca^{2+} (as measured by fura-2). The ratio of Ca^{2+} released from the store(s) to that of Ca^{2+} entered from the extracellular space was 1 : 2.5.

Intracellular Ca^{2+} release from the store(s) induced by cholecystokinin might be coupled with IP_3 formation, since saponin-permeabilized chief cells retained their ability to react to cholecystokinin with Ca^{2+} release by acting in concert with IP_3 [12]. Chew and Brown [10] suggested that the release of Ca^{2+} from the store(s) responding to cholecystokinin appeared to be mediated by IP_3 and to be utilized in exerting initial pepsinogen secretion. The initially evoked entry of Ca^{2+} from the extracellular space induced by cholecystokinin or ionomycin (as measured by fura-2) may not contribute to the initial pepsinogen secretion, since

the chelation of medium Ca^{2+} by EGTA did not influence the initial response. It, however, does play a role in producing sustained pepsinogen secretion combined with a late and more localized entry of Ca^{2+} from the extracellular space. The photoprotein, aequorin, which is known to be a detector of local elevation of $[Ca^{2+}]_i$ [14], could also detect the increase in $[Ca^{2+}]_i$ in response to cholecystokinin. The resting $[Ca^{2+}]_i$ measured by aequorin was at least 1 log unit higher than that measured by fura-2, thus indicating a different homeostasis and heterogeneity of $[Ca^{2+}]_i$ in the chief cell. This is not due to increased permeability of the aequorin-loaded cells as previously described. This was further supported by the fact that the TPA (or OAG)-mediated Ca^{2+} transient was detected only with aequorin measurement, and may be too localized to be detected by fura-2, and that the signal mode of the ionomycin-induced Ca^{2+} transient was different between aequorin and fura-2. The ionomycin (1 μM)-induced sharp decline in the aequorin signal after the brisk upstroke to a peak of the signal might reflect the rapid Ca^{2+} diffusion towards the cytosol without promoting energy-dependent Ca^{2+} removal, since the ionomycin-induced increase in $[Ca^{2+}]_i$ throughout the cytosol was maintained for a long time, even after the peak formation of the fura-2 signal. The intracellular source of the TPA-(or OAG)-sensitive and aequorin-detectable Ca^{2+} transient has not been identified. However, the elevation of $[Ca^{2+}]_i$ responding to TPA or OAG shares Ca^{2+} metabolic pathway(s) in common with cholecystokinin via intracellular Ca^{2+} release from the unidentified store(s) and the subsequent sustained increase in $[Ca^{2+}]_i$ due to the Ca^{2+} entering from outside the cells. The different mode of $[Ca^{2+}]_i$ returning to the resting level state in response to either cholecystokinin, TPA or OAG, monitored by aequorin, suggests that the local elevation of $[Ca^{2+}]_i$ is maintained even after the ATP-promoted Ca^{2+} removal by the Ca^{2+} store(s) or plasmalemma. This sustained but local elevation of $[Ca^{2+}]_i$ is due to the Ca^{2+} entering from the extracellular space as reflecting sustained pepsinogen secretion which requires medium Ca^{2+}.

Intracellular Ca^{2+} store(s)

In our X-ray spectra the secretory granules in the resting state had high sulfur concentration. The S content of the granules is probably due to an accumulation of sulfated anion and/or sulfated peptidoglycan and may be related to the condensation and/or aggregation of the secretory proteins [33]. The secretory granules in the resting state contained a higher amount of Ca relative to that of the cytoplasm. Most of the Ca seemed to be in a bound form, because the Ca level was too high for free Ca^{2+}. The ionomycin stimulation led to an increase in cytosolic Ca concentration and a decrease in Ca concentration in the secretory granules, suggesting that the Ca pool in the chief cell is located in the secretory granules or peripheral endoplasmic reticulum just beneath the apical surface associated with microtubular-microfilamentous system [12]. However, we could not identify the precise location of the Ca^{2+} pool(s), since the abundant secretory granules extending into the apical surface hid the peripheral endoplasmic reticulum. It must be considered that the endoplasmic reticulum at the peripheral cytoplasm just beneath the plasma membrane may be more sensitive to IP_3 stimulation, since IP_3 is formed in the plasma membrane and its concentration may be higher in the peripheral cytoplasm. This possibility is consistent with the fact that not all putative endoplasmic reticulums are sensitive to IP_3 [44] and another regulator such as GTP release Ca^{2+} from the store(s) [45], whose sensitive site(s) reside(s) in an intracellular compartment different from that of IP_3 [46].

The cholecystokinin or IP_3-induced Ca^{2+} release from the store(s) might be regulated by the function of the microtubular-microfilamentous system, since the pretreatment of cells with colchicine or cytochalasin D eliminated the Ca^{2+} release from the store(s) and the following initial pepsinogen secretion [12]. Therefore, the translocation and/or migration of the Ca^{2+} pool(s) into the lumen in the apical portion by regulating the cytoskeletal assembly may be a prerequisite for causing intracellular Ca^{2+} release from the store(s) and the subsequent exocytosis. Hence, the cytoskeletal assembly after the cell stimulation may act as a primary driving force for Ca^{2+} mobilization. Another possibility is that membrane tension is coupled to the Ca^{2+} channel(s) in the plasma membrane or in the endoplasmic reticulum, which

is constructed by cytoskeletal strands forming a viscoelastic coupling [47]. This study also exhibited the possibility that the pool which releases Ca^{2+} (by IP_3) and takes up Ca^{2+} (by a calmodulin-regulated Ca^{2+} pump) is the same or similar and that the IP_3-sensitive Ca^{2+} pool is a subset of the ionomycin-sensitive one. In the chief cell, the Ca^{2+}-removal system(s) in the Ca^{2+} pool(s) is dominant over that in the plasma membrane [12]. The directional Ca^{2+} cycling of the chief cell (Ca^{2+} shuttle) is different from that of the parietal cell (Ca^{2+} circuit) [21].

The X-ray microprobe data also suggest that the ionomycin stimulation produces an increase in permeability across the plasma membrane to Na^+, Cl^- and K^+, resulting in entry of Na^+ and Cl^- into the cell and efflux of K^+ from the cell. In addition, the ionomycin stimulation induced an increase in Na^+ and Cl^- concentrations in the secretory granules. This evidence suggests that the increase in cytoplasmic Na^+ (and Cl^-) by stimulation could bring about an increase in Na^+–Ca^{2+} exchange in the membrane of the intracellular Ca^{2+} store and an uptake of Na^+ (and Cl^-) into the Ca^{2+} pool as a counter-ion of released Ca^{2+}. This was supported by the finding that increasing the concentration of extravesicular Na^+ (40–100 mM), but not that of K^+, led to a release of Ca^{2+} from the submicrosomal vesicles that had been accumulated by ATP. It has been demonstrated that Na^+ induces the increase of membrane permeability to Ca^{2+}, resulting in an increase in reverse leakage of Ca^{2+} [34]. In addition, Na^+ and Ca^{2+} compete for binding sites at the intravesicular surface [35]. These results raise one possibility that the release of Ca^{2+} from the store(s) may be mediated by an Na^+–Ca^{2+} exchanger. In this case, it seems that the entry of external Na^+ into the cell induces Na^+–Ca^{2+} countertransport in the membrane of the Ca^{2+} pool(s), resulting in release of stored Ca^{2+}. Cytoplasmic alkalinization coupled to Na^+–H^+ exchange induces Ca^{2+} mobilization in platelets and increasing the intracellular pH changes the apparent affinity of IP_3 towards the Ca^{2+} store in permeabilized platelets, suggesting that increasing the concentration of cytosolic Na^+ plays a role in exerting Ca^{2+} mobilization in other types of cell [36,37]. But this was not the case, since elimination of Na^+ from the medium did not influence the cholecystokinin-elicited increase in $[Ca^{2+}]_i$ (see Fig. 3), thereby indicating that in the gastric chief cell Ca^{2+} mobilization is the first event after cell stimulation. Hence, increased Ca^{2+} in the cytosol (by ionomycin) might induce Na^+ and Cl^- entries and K^+ efflux, possibly by opening specific channels, resulting in an increase in Na^+ and Cl^- and a decrease in K^+ in the cytosol. A decrease in K^+ in the cytoplasm may relate to the membrane hyperpolarization as observed with acinar cells of the salivary glands [29]. Maruyama and Petersen [38] suggested that gastrin, cholecystokinin and acetylcholine activate two ion channels via changes in $[Ca^{2+}]_i$: one being a cation channel, permeable to Na^+ and K^+, and the other a specific K^+ channel. Hence, early K^+ efflux from the cells is triggered by an increase in $[Ca^{2+}]_i$.

Sustained pepsinogen secretion and Ca^{2+} mobilization

Since long-term Ca^{2+} chelation by EGTA depletes intracellular Ca^{2+} store(s), the exact role of Ca^{2+} metabolism and sustained pepsinogen secretion has not yet been substantiated in the case of EGTA. However, the Ca^{2+} entry blocker, lanthanum, inhibited sustained pepsinogen secretion (induced by cholecystokinin) in a dose-dependent fashion, suggesting that Ca^{2+} entry from the extracellular space is a prerequisite for exerting a sustained response. The prolonged increase in $[Ca^{2+}]_i$ that is induced by TPA, OAG or cholecystokinin, monitored by aequorin, is due to Ca^{2+} entering from the extracellular space. The activation of protein kinase C induced by TPA or OAG may thus require local Ca^{2+} mobilization that comprises two components: a rapid mobilization of intracellularly stored Ca^{2+} and a more prolonged influx of extracellular Ca^{2+}. Another possibility is that the increase in $[Ca^{2+}]_i$ induced by TPA or OAG could result from the action of protein kinase C rather than being required for its activation. A local but sustained Ca^{2+} entry from outside the cells induced by TPA might reflect the brisk Ca^{2+} removal system in the plasma membrane exceeding Ca^{2+} diffusion throughout the cytosol, since TPA enhances a Ca^{2+} efflux from the cell (local Ca^{2+} cycling) [43]. Thus local Ca^{2+} cycling just beneath the plasma membrane might

be required for prolonged activation of protein kinase C. On the other hand, the Ca^{2+} entered from the extracellular space in an initial phase might be used for activation of a variety of enzyme steps and for assembly of the microtubular-microfilamentous system rather than being required for activation of protein kinase C. The secretory event in exocytotic cells is an initial secretion from the stored or performed granules. With continued stimulation, the events trigger new synthesis and the continued secretion of enzyme [39]. Taken together, our data suggest that the initial component of pepsinogen secretion is associated with a high, fast and more global rise of cytoplasmic Ca^{2+} that is mediated by IP_3-elicited Ca^{2+} release from the store(s) and receptor-regulated Ca^{2+} entry from the extracellular space, and the sustained component with a late, sustained and more localized elevation of cytoplasmic Ca^{2+} depending upon influx of Ca^{2+} from the extracellular space that is mediated by diacylglycerol. We also suggest that the stimulation-sensitive Ca^{2+} pool(s) is (are) in or near the restricted apical cytoplasm and that this (these) pool(s) is (are) the same or similar to the ATP-dependent and calmodulin-regulated pool(s) connected with the cytoskeletal strands in the gastric chief cell.

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