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The cholecystokinin-induced Ca^{2+} shuttle from the inositol trisphosphate-sensitive and ATP-dependent pool, and initial pepsinogen release connected with cytoskeleton of the chief cell

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In guinea pig chief cells, inositol 1,4,5-trisphosphate (IP_3) caused release of Ca^{2+}, which was accumulated by ATP, from an endoplasmic reticulum-enriched fraction in both the permeable system and the cell-free system. This was mimicked with the Ca^{2+} ionophores A23187 and ionomycin on a large scale since an IP_3-sensitive Ca^{2+} pool might be a subset of the Ca^{2+} ionophore-sensitive Ca^{2+} pool. The permeable chief cells, but not the cell-free system, retained the ability to react to synthetic cholecystokinin octapeptide (CCK-OP) with Ca^{2+} release from an IP_3-sensitive pool due to of the non-additive but constant effect in exerting Ca^{2+} release from the store(s) induced by the combination with IP_3 and CCK-OP. The increase in the cytosolic free Ca^{2+} concentration of intact chief cells responding to CCK-OP or the Ca^{2+} ionophore, ionomycin, comprised two components, namely, that by the Ca^{2+} entry from the extracellular space, and that by the Ca^{2+} release from the intracellular space(s) (as measured by fura-2). When CCK-OP or ionomycin was added, there was a biphasic response of pepsinogen secretion. An initial but transient response reaching a peak in 5 min was followed by a sustained response reaching a peak in 30 min. The initial pepsinogen release was independent of medium Ca^{2+}, whereas the sustained one was dependent on medium Ca^{2+}. The results suggest that the intracellular Ca^{2+} release from the store(s), presumably endoplasmic reticulum, may trigger the initial pepsinogen release, whereas the sustained pepsinogen secretion may be caused by acting in concert with the initial response and external Ca^{2+} entry. On the other hand, the disruption of the microtubular-microfilamentous system by cholchicine or cytochalasin D failed to cause the Ca^{2+} release evoked by either IP_3, CCK-OP or Ca^{2+} ionophores and to cause the CCK-OP- or ionomycin-induced initial pepsinogen release. These findings suggest that the IP_3-sensitive pool is the same Ca^{2+} store which is completely or partially sensitive to CCK-OP and Ca^{2+} ionophores, respectively, and that the assembly of the cytoskeletal system is involved in initial intracellular Ca^{2+} metabolism and the following initial pepsinogen release. The assembly of the cytoskeletal system may be an early event in mediating the CCK-OP-induced initial pepsinogen release, perhaps by causing the Ca^{2+} release from an IP_3-sensitive pool of the chief cell. The translocation or attachment of the IP_3-sensitive pool brought about by cytoskeletal system might be necessary to cause Ca^{2+} release after the cell stimulation with CCK-OP.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′tetraacetic acid; fura-2 acetoxymethyl ester, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester; CCK-OP, synthetic cholecystokinin octapeptide.

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Introduction

Ca^{2+} and cyclic AMP play key roles in many cells used to study the stimulus-secretion coupling model [1,2]. Among others, the pepsinogen release from the chief cell is regulated by Ca^{2+} following the stimulation of either a peptidergic (cholecystokinin (CCK)) or cholinergic pathway besides the cyclic AMP synthesis that is evoked by either some peptidergic (secretin and vasoactive intestinal polypeptide) or β-adrenergic pathway [3–7]. The pepsinogen secretion that is induced by Ca^{2+}-mobilizing hormone is biphasic in some mammals; that is, the initial and transient pepsinogen release is followed by a sustained pepsinogen secretion [8–10]. Especially, the initial pepsinogen release may be caused by the Ca^{2+} release from an unidentified but inositol trisphosphate (IP_3)-sensitive pool, since the initial one evoked by these Ca^{2+}-mobilizing hormones (cholecystokinin and acetylcholine) is independent of extracellular Ca^{2+}, in contrast with that observed in sustained pepsinogen secretion, and these Ca^{2+}-mobilizing hormones induce IP_3 production [8–10].

On the other hand, the possibility that the assembly of the microtubules will be necessary in the onset of pepsinogen release has been suggested in bull-frog gastric mucosa, since the microtubular disrupting agents, colchicine and vinblastine, inhibited pepsinogen and acid secretory rates [11]. However, the characterization of the IP_3-sensitive Ca^{2+} pool and the physiological roles of the cytoskeletal system and of Ca^{2+} release in the onset of the initial pepsinogen release have not been substantiated. In order to elucidate the existence of the IP_3-sensitive Ca^{2+} pool and the roles of the cytoskeletal system and of Ca^{2+} release in the onset of the pepsinogen release, this study describes the mechanism of the intracellular Ca^{2+} release and the initial pepsinogen release connected with cytoskeleton of the guinea pig chief cell that are brought about by IP_3, Ca^{2+} ionophores and cholecystokinin octapeptide (CCK-OP), as assessed by the use of permeabilized cells, of intact cells and by subcellular fractionation.

Materials and Methods

Preparation of isolated chief cells

Dispersed heterogeneous gastric mucosal cells from a male guinea pig (Hartley strain, 300 g) were prepared by a previously described method [12,13], which was a modification from Berglindh [14]. Mucosal cells-containing $5 \cdot 10^6$ chief cells in 1.5 ml of Hanks' balanced-salt solution (Hanks' BSS) (parietal cells, 40%; chief cells, 40%; unidentified mucosal cells and red blood cells, 20%) obtained by enzymatic (collagenase and dispase) and chemical (EDTA) digestions were mixed with 3.75 ml of 90% Percoll solution (3.375 ml of 100% Percoll plus 0.375 ml of 10-fold concentrated Hanks' BSS) and 3.75 ml of oxygenated Hanks' BSS (pH 7.4) in the polycarbonate round-bottom tube. The final concentration of Percoll in 9 ml cell suspension was 37.5%. Cell separation by Percoll density gradient was effected by ultracentrifugation ($30\,000 \times g$, 15 min at 4° C) using a 28° C fixed-angle rotor [5,15]. After centrifugation, eight distinct cell bands were clearly visible. The density gradient was determined by measuring the distance from the meniscus to the colored bands formed by density marker beads. Chief cells, which were identified by their size (diameter, 10–12 μm) and their pepsinogen contents, were evenly distributed near the bottom from the meniscus (density, 1.062–1.076 g/ml). Parietal cells (diameter, 18 μm) were distributed near the interface (density 1.043–1.050 g/ml). Unidentified small mucosal cells and red blood cells appeared in the highest region (density, 1.034 g/ml) and the lowest region (density, 1.097 g/ml), respectively. The pepsinogen content of the fraction enriched in chief cells was 150.40 ± 29.58 $\mu g/10^6$ cells (six determinations), distinguishable from that of parietal cells (20.15 ± 0.48 $\mu g/10^6$ cells, four determinations). A chief cell-enriched fraction (2 ml) obtained by Percoll density gradient centrifugation was diluted in 40 ml oxygenated RPMI-1640 medium and was centrifuged ($350 \times g$ for 10 min at 4° C) to remove the Percoll from the cell suspension. The resultant pellet (chief cells) was washed in oxygenated RPMI-1640 medium (10^7 cells/10 ml) containing 10 mM of Hepes (pH 7.4). The abundance of chief cells was 80.78 ± 2.69% (ten determinations). The viability of separated chief cells (determined by

the exclusion of 0.4% Trypan blue) was 95%. The recovery of chief cells after applying the Percoll density gradient was $65.55 \pm 16.67\%$ (eight determinations) compared with that of dispersed mucosal cells. The amount of chief cells obtained from one guinea-pig gastric mucosa was almost $1.5 \cdot 10^7$. In Fig. 1 and Table I, cell separation was accomplished by use of the Beckman J2-21 elutriation system and the JE-6B elutriation rotor (Beckman, U.S.A.) (flow rate, 25 ml/min; centrifugal speed, 2000 rpm) [13]. The abundance and viability of the chief cells separated by the Beckman elutriation system were almost similar to those isolated by Percoll density gradient centrifugation.

Subcellular fractionation

Isolated chief cells (10^7 cells) were homogenized (30 strokes at 1500 rpm) at 0 °C in 2 ml of 0.32 M sucrose buffered with 5 mM Tris-maleate (pH 7.4) in a Teflon-glass homogenizer. The homogenate was spun at $2000 \times g$ for 10 min, and the resultant supernatant (post-nuclear fraction) was spun at $20000 \times g$ for 20 min (mitochondrial fraction). The last supernatant was spun at $100000 \times g$ for 1 h (microsomal fraction). The $100000 \times g$ pellet was applied to a 0.25–1.23 M sucrose linear gradient containing 15 mM CsCl and was centrifuged again at $100000 \times g$ for 1 h. The rough endoplasmic reticulum-enriched fraction obtained appeared at the bottom of the 1.23 M sucrose. The smooth endoplasmic reticulum-enriched fraction obtained appeared in the interface of the 0.25 M sucrose. An aliquot (100 μg protein) of either the mitochondrial, microsomal or endoplasmic reticulum vesicles was suspended in an incubation buffer consisting of 100 mM KCl, 4.5 mM $MgCl_2$, 1.0 μM $CaCl_2$ (prepared in EGTA buffer), 20 mM oxalate and 1.0 μCi $^{45}Ca^{2+}$ 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, the reaction was terminated by the addition of 2 ml of ice-cold 'stop solution'-containing 1 mM EGTA which was basically the same as the incubation medium, though without isotope $^{45}Ca^{2+}$. Separation of the isotope-containing vesicles from the incubation medium was achieved by filtration (Saltorius, pore size 0.3 μm) under mild suction. The dried filter pads were digested by 8 m of Aquazol and the $^{45}Ca^{2+}$ contents in the vesicles were counted by an Alloka liquid scintillation spectrometer. The marker enzymes corresponding to each subcellular fraction were not determined. However, the functional difference in Ca^{2+} uptake between the microsome (cholesterol-rich) and the endoplasmic reticulum vesicles (cholesterol-poor) was distinguished from their sensitivity to saponin or oxalate [16–18]. The ratio of cholesterol (mol)/phospholipid (mol) in endoplasmic reticulum vesicles was 1.385 ± 0.075 ($n = 6$), which differed from that taken by microsomes (cholesterol (mol)/phospholipid (mol) = 2; $n = 2$), as measured by Bartlett procedure (phospholipid) [20] and by using $FeCl_3/H_2SO_4$ (cholesterol) [21]. The protein quantity was determined by the method of Lowry et al. [19] using bovine serum albumin as the standard. The ATP-dependent $^{45}Ca^{2+}$ uptake by vesicles was expressed as the value with ATP minus that without ATP. The release of $^{45}Ca^{2+}$ from the endoplasmic reticulum vesicles was determined as follows. The endoplasmic reticulum vesicles were loaded with $^{45}Ca^{2+}$ by ATP for 20 min at 37 °C as described above, and the $^{45}Ca^{2+}$ uptake was stopped by 1 mM EGTA. Then either IP_3 (5 μM), ionophore A23187 (10 μM) or CCK-OP (10^{-8} M) was added and the $^{45}Ca^{2+}$ contents remaining in the vesicles after 5 min were counted as described above.

Preparation of saponin-treated chief cells

Isolated chief cells (10^6/ml, 4.5 mg protein/ml) purified by isopycnic centrifugation on linear density gradient of Percoll were immediately resuspended in a medium resembling the 'cytosol buffer' which contained the following composition; 20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 0.2 mM NaH_2PO_4, 0.8 mM Na_2HPO_4 and 25 mM $NaHCO_3$ in 15 mM Hepes buffer at pH 7.2. The medium also contained 2% bovine serum albumin, 50 μg/ml saponin, 10 μM antimycin and ATP-regenerating system consisting of 5 mM creatine phosphate and 50 μg/ml creatine phosphokinase. After an incubation period of 20 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, 0.49 mM $CaCl_2$). The medium Ca^{2+} concentration was fixed at about 180 nM by

EGTA buffer, as previously described [22]. In order to inhibit any mitochondrial Ca^{2+} metabolism, 10^{-5} M 2,4-dinitrophenol, in addition to antimycin, was added to the incubation medium. In Fig. 2 and Fig. 3 (left), after addition of 1.5 mM ATP to a cell suspension containing 1.0 μCi $^{45}Ca^{2+}$ (spec. act. 24.6 mCi/mg; New England Nuclear, U.S.A.), IP_3 (5 μM), A23187 (10 μM) or CCK-OP (10^{-8} M) was added at 20 min. The final reaction was stopped by adding 2 ml of the same 'cytosol buffer' without isotope $^{45}Ca^{2+}$. The cell suspension was placed on a Millipore filter (RAWP, pore size 1.2 μm) under mild suction (4.9 inches Hg). The cell suspension on the filter was washed four times with 2 ml of 'cytosol buffer' and the dried filter pads were digested by 200 μl of distilled water and 500 μl of Protosol for 12 h. After adding 8 ml of Aquazol, samples were counted for radioactivity in an Alloka liquid scintillation spectrometer using the ^{14}C channel.

Measurement of cytosolic Ca^{2+} concentration

The measurement of $[Ca^{2+}]_{cyt}$ was done by fura-2 loaded cells [23]. 2 μM fura-2 acetoxymethyl ester (which was diluted from 1 mM by dimethyl sulfoxide to obtain a final concentration of 2 μM) 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. The cell suspension was incubated for 15 min at 37°C in a 95% O_2/5% CO_2 chamber (pH 7.4). After loading of cells with fura-2, the cell suspension was washed twice by 40 ml of RPMI-1640 medium and 10^6 cells were resuspended in 2 ml of Krebs-Ringer bicarbonate buffer in the presence (1.3 mM Ca^{2+}) or absence of medium Ca^{2+} (prepared by omitting $CaCl_2$ and by adding 1 mM EGTA). 10 μl of 200 mM EGTA was added when the cells were transferred in a cuvette just before assay. The fura-2 loaded cell suspension in a cuvette was preincubated at 37°C for 1 min and its fluorescence was read for 5 min with stirring after stimulation with secretagogues.

The fluorescence was recorded with a Hitachi 650-60 fluorescence spectrometer (Tokyo, Japan). The excitation and emission wavelengths were 335 and 500 nm with 10 and 20 bandwidths, respectively. $[Ca^{2+}]_{cyt}$ was calculated using the formula [23]; $[Ca^{2+}]_{cyt} = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant of fura-2 for Ca^{2+} (224 nm). Calibration of fura-2-Ca^{2+} signal was made by adding 4 mM of EGTA from a 200 mM of stock solution in Tris-base (pH 8.3), followed by 0.1% Triton X-100 (F_{min}) and by 4 mM of $CaCl_2$ (F_{max}). When medium Ca^{2+} was zero (1 mM of EGTA), 3 mM of EGTA was added. F is the relative fluorescence measurement of the sample. The ratio F_{max}/F_{min} was 3.04 ± 0.18 (medium Ca^{2+}, 1.3 mM), (14 determinations) and 3.31 ± 0.15 (0 mM medium Ca^{2+} plus 1 mM EGTA) (14 determinations), respectively. Subsequent addition of EGTA did not change the fluorescence, indicating that fura-2 was accumulated in the cells. In Fig. 3 (right), saponin-permeabilized cells (10^7 cells) were loaded by 2 μM of fura-2 acetoxymethyl ester in 10 ml 'cytosol buffer' under the same conditions as described above. The changes in fluorescence after the addition of ATP (1.5 mM) and the subsequent addition of ionomycin (5 μM) were measured.

Measurement of pepsinogen release

Pepsinogen activity was measured by the method of Anson and Mirsky [24]. Pepsinogen secretion from chief cells was measured on isolated gastric glands without cell purification by Percoll density gradient or Beckman elutriation, which were composed of approximately equal proportions of chief and parietal cells, since pepsinogen secretion, but not Ca^{2+} metabolism, reflects the function solely of chief cells in a heterogeneous cellular preparation. Gastric glands (10^6 chief cells/600 μl) were incubated in oxygenated Krebs-Ringer bicarbonate buffer, containing 0.2% glucose, at 37°C with (1.3 mM Ca^{2+}) or without medium Ca^{2+} (0 mM Ca^{2+} plus 1 mM EGTA). EGTA was added to the cell suspension just before incubation. At an appropriate time after addition of CCK-OP or ionomycin, the cell suspension was centrifuged at $10000 \times g$ for 30 s and the resultant supernatant was aspirated and stocked. The pellet was resuspended in 600 μl of the same medium and sonicated for 30 s. 100 μl of either supernatant or 20-fold diluted pellet by the same incubation medium were added to 400 μl acidic solution (320 μl H_2O and 80 μl 0.3 M HCl) containing 2.5% human hemoglobin and then incubated for 10 min at 37°C. The reaction was

stopped by adding 1 ml of 5% trichloroacetic acid. The cell suspension was centrifuged at $750 \times g$ for 10 min and the absorbance of the supernatant (500 μl supernatant in 2.5 ml 0.5 M Na_2CO_3 plus 250 μl 0.1 M phenol reagent) was read at 640 nm using tyrosine as the standard. An appropriate blank, in which trichloroacetic acid was added before the sample, was run in parallel. Pepsinogen release was calculated as a percentage of total pepsinogen activity present in the cells plus that in the medium.

Treatment of cells with colchicine and cytochalasin D

A cell suspension (10^6 chief cells) consisting of either intact cells or permeable cells which was suspended in Krebs-Ringer bicarbonate buffer and 'cytosol buffer', respectively, was preincubated with colchicine (10 μg/ml) or cytochalasin D (10 μg/ml) for 5–10 min at 37°C prior to the measurements of Ca^{2+} flux and pepsinogen secretion.

^{125}I-cholecystokinin octapeptide binding assay

A cell suspension (10^6 chief cells) in 1 ml of oxygenated Hanks' BSS with or without medium Ca^{2+} was incubated with 0.05 μCi (22.7 pM) of ^{125}I-labeled CCK-OP (spec. act. 2200 Ci/mmol, New England Nuclear, U.S.A.) at 37°C. At an appropriate time, the cell suspension was centrifuged at $10000 \times g$ for 30 s to separate bound from free hormone and radioactivity in the resultant pellet was counted by an Alloka gamma counter. Specific binding was expressed as the value without nonradiolabeled CCK-OP (total binding) minus that with excess nonradiolabeled CCK-OP (10^{-8} M) (nonspecific binding).

Measurement of inner-membrane bound Ca^{2+} release

50 μM chlorotetracycline suspended with Tris-saline (pH 7.4) was added to a chief cell suspension (10^7 cells/10 ml) in RPMI-1640 medium (pH 7.4), and then cells were loaded for 30 min at 37°C. Dye-loaded 10^6 cells were resuspended with 2 ml of Ca^{2+}-poor medium (Hepes-Tyrode's solution) without EGTA. 20 μl 100 mM $CaCl_2$ (to obtain a final concentration of 1 mM) were added to the cell suspension, which was then left for 20 min at 24°C to equilibrate cellular Ca^{2+} contents. Fluorescence was recorded by a Hitachi 650-60 (excitation, 400 nM; emission, 530 nM) at 24°C with continuous stirring. The fluorescence change after the stimulation is expressed as in arbitrary units.

Materials

The sources of some of the above-mentioned reagents have been quoted previously [12,13]. Inositol 1,4,5-trisphosphate, antimycin, 2,4-dinitrophenol, creatine phosphate, creatine phosphokinase, human blood hemoglobin, cytochalasin D (from *Zygosporium mansoniee*), saponin and cholecystokinin-octapeptide 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 was obtained from Dougindo (Japan).

Results

Ca^{2+} flux in cell-free system

Table I shows the ATP-dependent $^{45}Ca^{2+}$ uptake by each subcellular fraction obtained from differential centrifugation and sucrose density gradient. The content of $^{45}Ca^{2+}$ uptaken by ATP in each subcellular fraction was expressed as nmol/mg protein per 20 min and was further converted to fmol/20 min in each subcellular fraction from a single chief cell calculated by protein quantity. The distribution ratio of ATP-dependent Ca^{2+}-removal system for each subcellular fraction per single chief cell of either postnucleus, mitochondria, microsome or endoplasmic reticulum-enriched fraction (endoplasmic reticulum fraction) was calculated to be 16.1 : 4.6 : 7.8 : 1, respectively). The ratio of net Ca^{2+} taken up to ATP utilized was about 2 : 1 for the endoplasmic reticulum fraction of the chief cells (not shown). The quantity of protein of endoplasmic reticulum vesicles from a single chief cell was 0.05 ng, which was 1.1% of that occupying the single chief cell (4.5 ng). The endoplasmic reticulum vesicles caused an ATP-dependent and time-dependent $^{45}Ca^{2+}$ uptake in the presence of 20 mM oxalate (Table I and Fig. 1) whose value (2.40 ± 0.15 fmol/cell per 20 min) was almost equal to that taken by plasmalemmal permeabilized (by saponin) and mitochondrial poisoned (by 2,4-dinitrophenol and

TABLE I

THE ATP-DEPENDENT Ca^{2+} UPTAKE IN EACH SUBCELLULAR FRACTION FROM ISOLATED CHIEF CELL

The concentrations of reagents used were as follows: 1.5 mM ATP; 1.0 μM CCCP; 100 μg/ml saponin; 20 mM oxalate. 100% corresponds to the ATP-dependent $^{45}Ca^{2+}$ uptake in endoplasmic reticulum without CCCP and saponin and with 20 mM oxalate. The data represent the means ± S.E. of the number of the samples in parentheses from two to five separate experiments as the values with ATP minus that without ATP.

subcellular fraction	protein per chief cell (ng)	cholesterol (mmol) /phospholipid (mmol)	ATP-dependent $^{45}Ca^{2+}$ uptake (f moles/cell/20min)	
post-nucleus	0.71		38.60±4.23 (n=15)	
mitochondria	0.48		11.04±1.43 (n=8)	
microsome	0.32	2 (n=2)	18.72±2.42 (n=8)	
endoplasmic reticulum	0.05	1.385±0.075 (n=6)	2.40±0.15 (n=8)	100%
			with CCCP	106.7% (n=2)
			with saponin	113.9±20.9 % (n=4)
			without oxalate	35.51% (n=2)

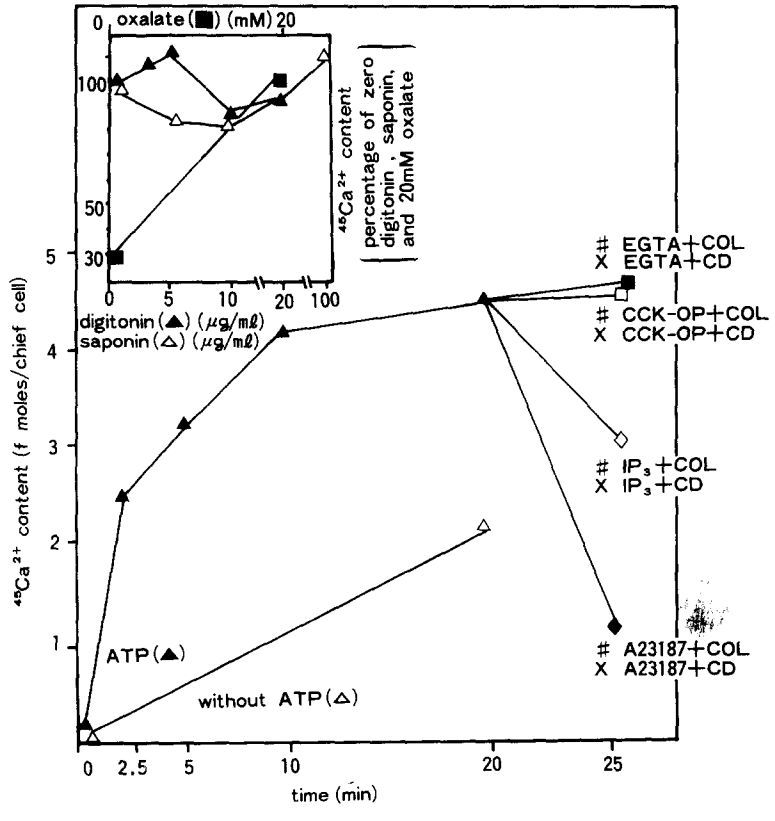

Fig. 1. Time-course of ATP-dependent $^{45}Ca^{2+}$ uptake into the endoplasmic reticulum-enriched vesicles of the chief cell. The concentrations of reagents used were as follows: 1.5 mM ATP; 1 mM EGTA; 5 μM IP_3; 10 μM A23187; 10 nM CCK-OP; 10 μg/ml colchicine (COL); 10 μg/ml cytochalasin D (CD). EGTA plus indicated reagents were added 20 min after ATP stimulation. Each point represents the mean from two to five separate experiments. (Inset). The effects of oxalate, digitonin and saponin on ATP-dependent $^{45}Ca^{2+}$ uptake into the endoplasmic reticulum-enriched vesicles. 100% corresponds to the ATP-dependent $^{45}Ca^{2+}$ uptake in both the absence of digitonin (saponin) and the presence of 20 mM oxalate. Each point represents the mean from four separate experiments.

antimycin) chief cell (see Fig. 2, 2.93 fmol/cell per 20 min with 20 mM oxalate, 0.85 fmol/cell per 20 min without oxalate) rather than post-nucleus, mitochondria, or microsome. In the absence of oxalate the endoplasmic reticulum vesicles caused an ATP-dependent $^{45}Ca^{2+}$ uptake that reached 0.85 fmol/cell per 20 min, being therefore similar to that taken by the permeable chief cell. The molar ratio of cholesterol phospholipid of endoplasmic reticulum vesicles was 69.3% of the ratio observed in microsomes, thus differing from that of the plasmalemmal-enriched fraction (see Ref. 25, cholesterol (mmol)/phospholipid (mmol); 1.95). Furthermore, the ATP-dependent $^{45}Ca^{2+}$ uptake in endoplasmic reticulum vesicles was dependent on oxalate, which is known to be a stimulator of Ca^{2+} uptake into the endoplasmic reticulum [26], while saponin, a disruptive agent of cholesterol-rich plasma membrane [27], and carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), an inhibitor of mitochondrial energy metabolism [28] failed to affect the ATP-dependent $^{45}Ca^{2+}$ uptake in endoplasmic reticulum vesicles (Table I and Fig. 1, inset). These observations might determine that one of the ATP-dependent Ca^{2+}-removal systems is located in endoplasmic reticulum fraction besides the plasmalemma and mitochondria. As shown in Fig. 1, the Ca^{2+} ionophore A23187 (10 μM) plus EGTA (1 mM) but not EGTA alone induced a rapid release of Ca^{2+} from endoplasmic reticulum vesicles, thus indicating that the Ca^{2+} accumulated by ATP exists in internal store of endoplasmic reticulum vesicles and is not bound to their exterior. IP_3 (5 μM) also caused a rapid release of Ca^{2+} from endoplasmic reticulum vesicles at about half of that evoked by A23187, thus indicating that endoplasmic reticulum vesicles in the chief cell might be at least the source of the IP_3-sensitive pool and that the pool which releases and takes up Ca^{2+} is the same or similar. The re-uptake of Ca^{2+} into endoplasmic reticulum vesicles by IP_3 plus ATP as shown in Fig. 2 (permeable cell) was not observed because of the chelation of Ca^{2+} by excess EGTA when Ca^{2+} was released from endoplasmic reticulum vesicles to the medium. CCK-OP (10^{-8} M) failed to cause $^{45}Ca^{2+}$ release directly from endoplasmic reticulum vesicles, in contrast with that observed in permeable cells (see, Fig. 2) and intact cells (see Fig. 4), indicating that the CCK-OP-

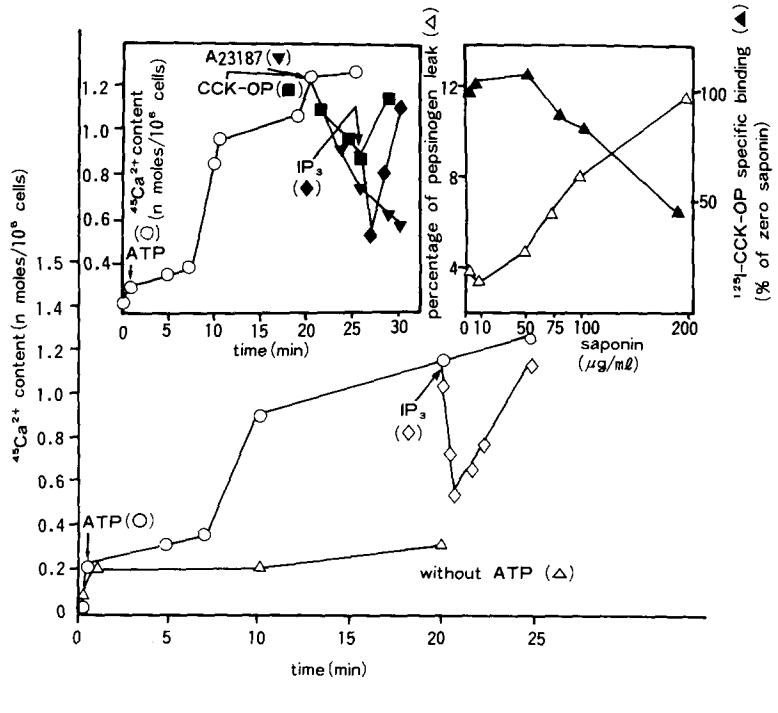

Fig. 2. $^{45}Ca^{2+}$ released by IP_3 that are accumulated by ATP in saponin-permeabilized chief cells. The concentrations of reagents used were as follows: 1.5 mM ATP; 5 μM IP_3; 10 μM A23187; 10 nM CCK-OP. IP_3 was added 20 min after ATP stimulation. The ATP-dependent $^{45}Ca^{2+}$ uptake in the presence of 20 mM oxalate was 2.93 nmol/10^6 cells ($n = 2$). The data represent the mean from two separate experiments (four determinations). (Inset, left) $^{45}Ca^{2+}$ released by CCK-OP, A23187 and the combination with CCK-OP plus IP_3. CCK-OP or A23187 was added at 20 min. Then IP_3 was added 5 min after the cell stimulation with CCK-OP. (Inset, right) The effects of saponin on non-stimulated pepsinogen leak and on ^{125}I-CCK-OP binding to chief cells. Pepsinogen leak was measured 20 min after saponin addition in the presence of medium Ca^{2+}. ^{125}I-CCK-OP binding was expressed as a specific binding at 20 min in the presence of medium Ca^{2+}. 100% corresponds to ^{125}I-CCK-OP specific binding in the absence of saponin. The data in insets represent the mean from two separate experiments (four determinations).

induced Ca^{2+} release from endoplasmic reticulum vesicles may require some cytosolic elements.

Ca^{2+} flux in permeable cells

Fig. 2 (inset, right) shows the saponin-induced pepsinogen leak from permeable chief cells. Treatment of 10^6 chief cells with 50 μg of saponin for 20 min at 37°C retained the ability to stock pepsinogen in the cell, while there was a leak of pepsinogen over the concentration of 75 μg of saponin. In addition, specific binding of ^{125}I-CCK-OP to chief cells' receptors was decreased by above 75 μg of saponin. Therefore, permeabilization of chief cells was accomplished by 50 μg of saponin per 4.5 mg protein (10^6 chief cells) for 20 min at 37°C.

As shown in Fig. 2, in saponin-permeabilized chief cells there was a rapid uptake of $^{45}Ca^{2+}$ by the following addition of ATP (1.5 mM) in the presence of ATP-regenerating system that reached a steady state after 25 min. This Ca^{2+} uptake might reflect the non-mitochondrial Ca^{2+}-removal system(s) because of its insensitivity to CCCP and antimycin. When 20 mM oxalate was added to the incubation medium, the ATP-dependent $^{45}Ca^{2+}$ uptake in permeable cells was increased by 2.93 fmol per single chief cell for 20 min whose value was very similar to that taken by endoplasmic reticulum vesicles in cell-free system (see, Table I). However, in the permeable system but not in the cell-free system, no oxalate was added to the incubation medium so that we could observe the IP_3-induced Ca^{2+} release and re-uptake. At 20 min, 5 μM of IP_3 was added, leading a 54% loss of cellular $^{45}Ca^{2+}$ contents due to intracellular Ca^{2+} release from the Ca^{2+} pool(s) (from 1.154 to 0.533 nmol/10^6 cells within 1 min). A decrease in $^{45}Ca^{2+}$ contents in permeable cells by IP_3 was transient, since there was a re-uptake of $^{45}Ca^{2+}$ in the presence of ATP-regenerating system during a 4 min period following the addition of IP_3 (from 0.533 to 1.128 nmol/10^6 cells). The Ca^{2+} ionophore A23187 (10 μM) caused Ca^{2+} release by 10 min (from 1.154 to 0.600 nmol/10^6 cells); however, there was no re-uptake of $^{45}Ca^{2+}$ by A23187, thus differing from the case of IP_3 (Fig. 2, inset, left). Since ^{125}I-CCK-OP could bind to 50 μg of saponin-treated chief cells, permeable chief cells might retain the ability to react to CCK-OP with Ca^{2+} release. When CCK-OP (10^{-8} M) was added to the incubation medium at 20 min, there was a 29.5% loss of $^{45}Ca^{2+}$ followed by re-uptake to the pre-stimulation value. When IP_3 (5 μM) was added after CCK-OP, there was a further $^{45}Ca^{2+}$ release and the sum of the $^{45}Ca^{2+}$ released by CCK-OP plus IP_3 was constant and was similar to that taken by IP_3 alone (Fig. 2, inset, left).

The effects of cytoskeletal disrupting agents on IP_3-induced Ca^{2+} release and ATP-dependent Ca^{2+} removal (permeable cell)

Colchicine is known to bind to free tubulin dimer and to inhibit microtubulus polymerization by reacting with the microtubule ends forming a colchicine-tubulin complex [29]. Cytochalasins, especially B, D and E, are known to interfere with contractile function of the microfilament [30,31]. Since secretagogue-induced acid secretion in the parietal cell was inhibited with a potency order cytochalasin D > E = B [32], cytochalasin D was employed in this study. The concentrations of colchicine and cytochalasin D used were according to those described for parietal cells [12,13]. As shown in Fig. 3 (left), pretreatment of permeable chief cells with colchicine (10 μg/10^6 cells per ml) or cytochalasin D (10 μg/10^6 cells per ml) for 5 min at 37°C prior to the stimulation with IP_3 (5 μM) inhibited IP_3-induced Ca^{2+} release (the loss of cellular $^{45}Ca^{2+}$ contents: IP_3 alone, 54%; IP_3 + colchicine, 28%; IP_3 + cytochalasin D, 28%) and the subsequent Ca^{2+} re-uptake. Especially, the subsequent re-uptake in the presence of ATP-regenerating system, found in the case of IP_3 alone, was completely abolished by colchicine and cytochalasin D. This was also observed with the Ca^{2+} measurement by fura-2 acetoxymethyl ester (Fig. 3, right). The resting level of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) of permeable chief cell was 243.39 ± 65.81 nM ($n = 5$) ($F_{max}/F_{min} = 1.728 \pm 0.076$, $n = 5$). Subsequent addition of ATP (1.5 mM) caused a substantial uptake of Ca^{2+} into the cellular store(s), resulting in a decrease in $[Ca^{2+}]_{cyt}$ (from 243.39 ± 65.8 to 172.11 ± 39.53 nM, $n = 5$). Subsequent addition of the Ca^{2+} ionophore, ionomycin (5 μM), caused a 27.1 nM final rise ($n = 2$) of $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release from the same or similar store(s) sensitive to ATP.

Ionomycin was used as a substitute for A23187

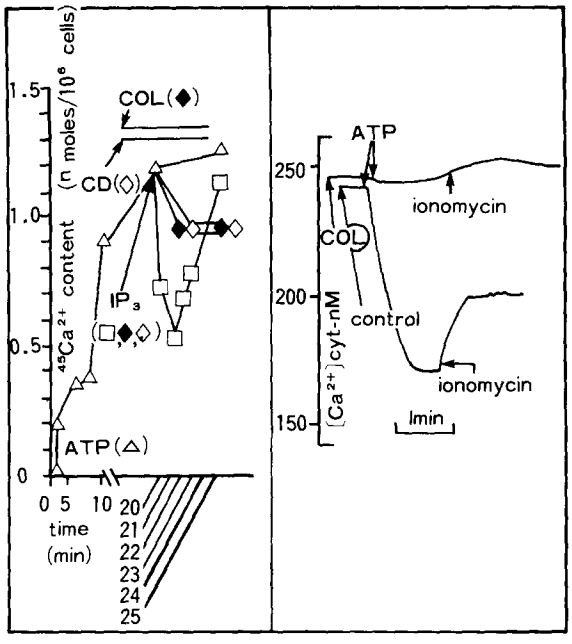

Fig. 3. The effects of colchicine (COL) and cytochalasin D(CD) on IP_3-induced $^{45}Ca^{2+}$ release (left) and on ATP-promoted Ca^{2+} removal (right) in saponin-permeabilized chief cells. The concentrations of reagents used were as follows: 10 μg/ml COL; 10 μg/ml CD; 1.5 mM ATP; 5 μM IP_3; 5 μM ionomycin. Colchicine or cytochalasin was added just 5 min before IP_3 (left) or ATP (right) was added. The data represent the mean from five separate experiments.

because of the inherent fluorescence of A23187. On the other hand, pretreatment of permeable chief cells with colchicine (20 $\mu g/10^6$ cells per 2 ml in a cuvette) for 5 min at 37°C prior to addition of 1.5 mM ATP prevented the removal of Ca^{2+} (by ATP) from the intracellular store(s) and the subsequent Ca^{2+} release by ionomycin. These results suggest that the assembly of the microtubular-microfilamentous system of the chief cell might be involved in IP_3-induced Ca^{2+} release and ATP-dependent Ca^{2+} removal (uptake and re-uptake).

Ca^{2+} flux in intact cell and the effects of cytoskeletal disrupting agents on CCK-OP or ionomycin-induced Ca^{2+} release

Fig. 4 shows the stimulation of $[Ca^{2+}]_{cyt}$ with 10^{-8} M CCK-OP or 5 μM ionomycin in the presence (1.3 mM Ca^{2+}) or absence (0 mM Ca^{2+} plus 1 mM EGTA) of medium Ca^{2+} as measured by fura-2 acetoxymethyl ester. The resting levels of $[Ca^{2+}]_{cyt}$ of the chief cell in the presence or absence of medium Ca^{2+} were 236.38 ± 69.97 nM ($n = 6$) and 123.64 ± 14.10 nM ($n = 6$), respectively. In the presence of medium Ca^{2+}, CCK-OP or ionomycin caused a rapid increase in $[Ca^{2+}]_{cyt}$ that reached 75 nM (from 236.38 ± 69.97 to 310.79 ± 12.29 ($n = 7$)) and 163 nM (to 399.72 ± 21.52 ($n = 5$)) final rise, respectively (Fig. 4, inset, left). The CCK-OP-induced $[Ca^{2+}]_{cyt}$ increase was transient, its mode being distinguishable from that induced by ionomycin. The decrease in $[Ca^{2+}]_{cyt}$ (induced by CCK-OP) may reflect either Ca^{2+} efflux across the plasma membrane, Ca^{2+} uptake into the pool(s) or both, perhaps due to pumps [33,34]. Ionomycin's failure to cause any decrease in $[Ca^{2+}]_{cyt}$ might reflect that the continuous Ca^{2+} entry exceeded either the Ca^{2+} efflux, the Ca^{2+} uptake or both, since in the absence of medium Ca^{2+}, a drop in $[Ca^{2+}]_{cyt}$ subsequent to the maximum rise in $[Ca^{2+}]_{cyt}$ brought about by ionomycin was observed. In Ca^{2+}-free medium with 1 mm EGTA, however, CCK-OP or ionomycin also caused an increase in $[Ca^{2+}]_{cyt}$ that reached 20 nM (from 123.64 ± 14.10 to 143.84 ± 5.45 ($n = 6$)) and 44 nM (to 168.03 ± 9.88 ($n = 7$)) final rise, respectively (Fig. 4). This slight but significant increase in $[Ca^{2+}]_{cyt}$ (by CCK-OP or ionomycin) in the absence of medium Ca^{2+} might be due to uncontaminated intracellular Ca^{2+} release from the store(s) as shown in permeable cells (see Figs. 2, 3), since the Ca^{2+} entry blocker, lanthanum (10^{-4} M) [35], did not affect the CCK-OP- or ionomycin-induced $[Ca^{2+}]_{cyt}$ increase that was independent of medium Ca^{2+} (CCK-OP: from 139.03 to 165.93 nM, 26.9 nM final rise, $n = 2$; ionomycin: to 177.83 nM, 38.8 nM final rise, $n = 2$), while the medium Ca^{2+}-dependent increase in $[Ca^{2+}]_{cyt}$ was almost completely inhibited by lanthanum due to blocking of the Ca^{2+} entry from the medium without inhibiting the Ca^{2+} release from the store(s) in the case of CCK-OP (from 192.24 to 224.00 nM, 31.8 nM final rise, $n = 2$), (Fig. 4, inset, left). The ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ that was dependent on medium Ca^{2+} (measured by fura-2) was partially inhibited by lanthanum (to 264.72 nM, 72.5 nM final rise) with medium Ca^{2+}. In the presence of medium Ca^{2+} the signal detected by chlorotetracycline was

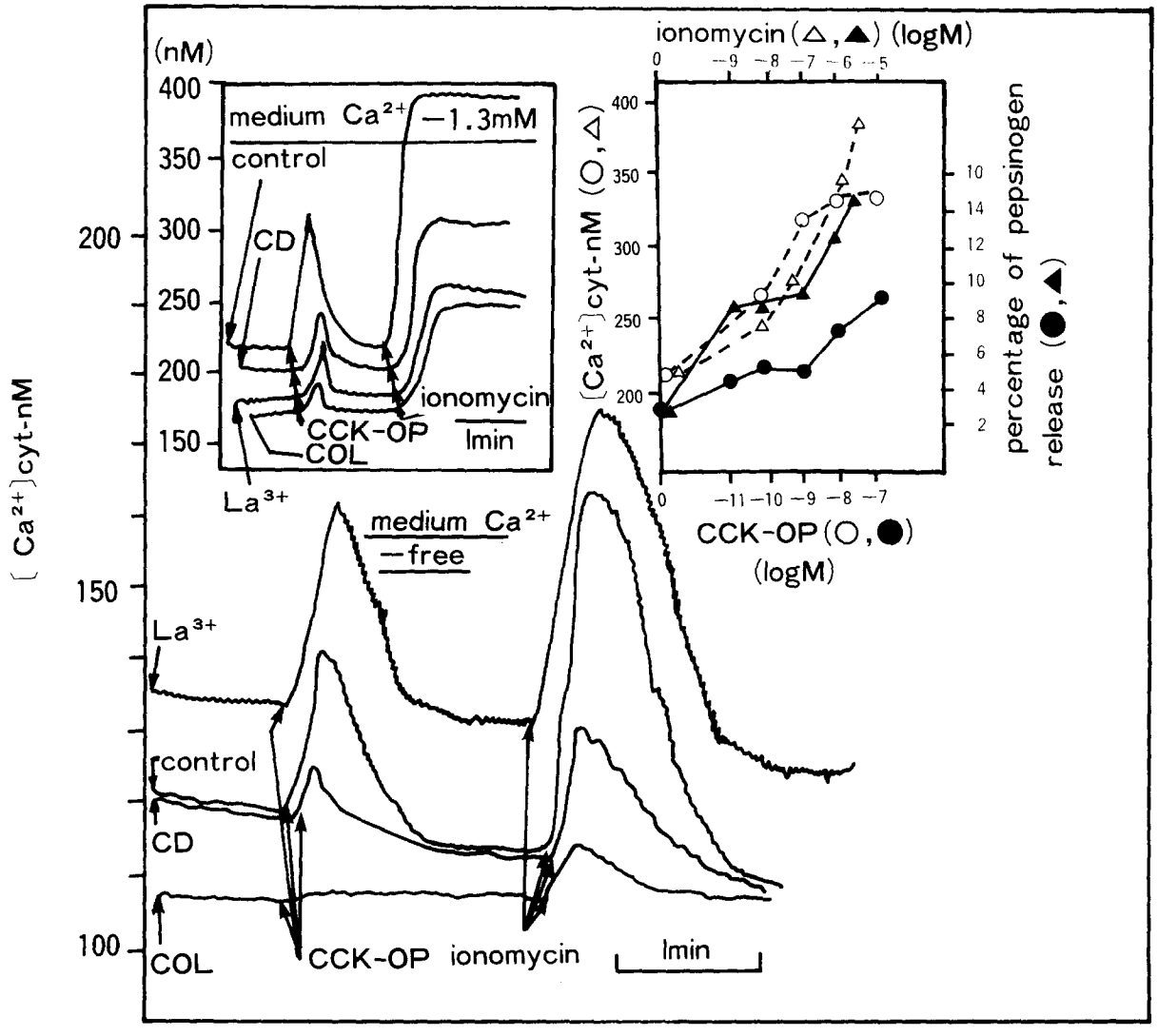

Fig. 4. The effects of colchicine (COL) and cytochalasin D(CD) on CCK-OP- or ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ in the absence of medium Ca^{2+} (0 mM medium Ca^{2+} plus 1 mM EGTA). The concentrations of reagents used were as follows: 10 μg/ml colchicine; 10 μg/ml cytochalasin D; 100 μM lanthanum (La^{3+}); 10 nM CCK-OP; 5 μM ionomycin. Colchicine, cytochalasin D or La^{3+} was added just 5 min before CCK-OP was added. La^{3+} caused cell aggregation, however, the responses to CCK-OP and ionomycin were maintained. The data represent the means from four separate experiments. (Inset, left) The effects of colchicine and cytochalasin D on CCK-OP- or ionomycin-induced $[Ca^{2+}]_{cyt}$ change in the presence of medium Ca^{2+} under experimental conditions identical to Fig. 4. The data represent the means from four separate experiments. (Inset, right) Dose-response curves for effects of CCK-OP and ionomycin on $[Ca^{2+}]_{cyt}$ changes and pepsinogen secretion. Pepsinogen secretion with the indicated agents was determined after 30 min incubation in the presence of medium Ca^{2+}. $[Ca^{2+}]_{cyt}$ with the indicated agents was determined 10 s after the cell stimulation in the presence of medium Ca^{2+}. The data represent the means from four to seven separate experiments. (These determinations were independent from those taken by Fig. 4 and Fig. 4 (inset, left).)

decreased upon ionomycin (1 μM) stimulation but not upon CCK-OP stimulation (arbitrary unit of fluorescence: from 50 to 25 within 1 min after the stimulation, $n = 2$). Lanthanum pretreatment did not affect the ionomycin-induced decrease in the chlorotetracycline signal (from 52 to 25, $n = 2$). The patchy fluorescence of the chlorotetracycline is assumed to arise from the inner membrane

because of its clearly peripheral localization [45]. Therefore, the lanthanum-insensitive increase in $[Ca^{2+}]_{cyt}$ induced by ionomycin might reflect inner-membrane-bound Ca^{2+} release besides the Ca^{2+} release from the store(s).

Pretreatment of intact chief cells with colchicine (20 $\mu g/10^6$ cells per 2 ml in cuvette) or cytochalasin D (20 $\mu g/10^6$ cells per 2 ml in cuvette) for 5 min at 37 °C prior to the stimulation failed to increase the $[Ca^{2+}]_{cyt}$ induced by CCK-OP or ionomycin which was independent of medium Ca^{2+} (percentage of inhibition: versus CCK-OP; colchicine, 100%, cytochalasin D, 66.5%; versus ionomycin; colchicine, 84.5%, cytochalasin D, 76.4%). The CCK-OP or ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ which had been pretreated with colchicine in the absence of medium Ca^{2+} was 0 nM final rise (from 109.80 ± 9.58 ($n = 3$) to 109.8 ($n = 2$) nM) and 6.8 nM final rise (to 116.63 ± 4.20 ($n = 3$) nM) respectively. The CCK-OP or ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ which had been pretreated with cytochalasin D was 6.7 nM final rise (from 122.92 ± 7.07 ($n = 5$) to 129.61 ($n = 2$) nM) and 10.4 nM final rise (to 133.28 ± 6.04 ($n = 4$) nM), respectively. These results suggest that the assembly of the microtubular-microfilamentous system of the chief cell might be involved in CCK-OP- or ionomycin-induced Ca^{2+} release as well as the IP_3-induced Ca^{2+} release. Colchicine and cytochalasin D also caused inhibition of the increase in $[Ca^{2+}]_{cyt}$ that was dependent of medium Ca^{2+} (Fig. 4, inset, left). The CCK-OP- or ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ pretreated with colchicine in the presence of medium Ca^{2+} was 2.57 nM final rise (from 178.68 ± 21.42 ($n = 4$) to 181.25 ± 14.08 ($n = 3$) nM) and 72.68 nM final rise (to 251.35 ± 21.58 ($n = 3$) nM), respectively. The CCK-OP- or ionomycin-induced increase in $[Ca^{2+}]_{cyt}$ pretreated with cytochalasin D in the presence of medium Ca^{2+} was 15.54 nM final rise (from 225.59 ± 59.7 ($n = 3$) to 241.13 ($n = 2$) nM) and 86.2 nM final rise (to 311.79 ± 17.33 ($n = 5$) nM), respectively. This suggests that the CCK-OP-induced external Ca^{2+} entry might be regulated by the cytoskeleton as well as the Ca^{2+} release from the store(s). It seems unlikely that the ionomycin-induced Ca^{2+} entry was affected by colchicine and cytochalasin D because of their slight inhibition when the medium Ca^{2+} was present.

The ionomycin-induced Ca^{2+} release including innermembrane-bound Ca^{2+} release was also inhibited by colchicine as measured by aequorin bioluminescence in the absence of medium Ca^{2+} (ionomycin (5 μM) alone: from 4.5 μM to 5.6 μM $[Ca^{2+}]_{cyt}$, ionomycin plus colchicine (10 $\mu g/10^6$ cells per ml); from 4.5 μM to 4.475 μM $[Ca^{2+}]_{cyt}$, not shown). On the other hand, colchicine and cytochalasin D failed to inhibit IP_3 or A23187-induced Ca^{2+} release from endoplasmic reticulum vesicles in the cell-free system (see, Fig. 1), suggesting that these inhibitory effects on intracellular Ca^{2+} release require some cytosolic elements in intact cells.

CCK-OP or ionomycin-induced pepsinogen secretion in intact cells

Fig. 5 shows the time-course of CCK-OP- (10^{-7} M) or ionomycin- (5 μM) induced pepsinogen secretion (and/or release) by chief cells originated in gastric glands. Since in the absence of medium Ca^{2+}, CCK-OP and ionomycin caused an initial but transient pepsinogen release, but no sustained response, it seems that the CCK-OP- or ionomycin-induced pepsinogen secretion in the presence of medium Ca^{2+} was a biphasic response, that is, initial but transient pepsinogen release followed by a sustained response. It is unlikely that the lack of sustained pepsinogen secretion (by CCK-OP) in the absence of medium Ca^{2+} is due to the inhibition of CCK-OP binding to its receptor by EGTA, since ^{125}I-cholecystokinin binding was not affected by the omission of medium Ca^{2+} (by EGTA) for 20 min (Fig. 5, inset). Therefore, the biphasic pepsinogen secretion might be factor explaining why an initial response (by CCK-OP or ionomycin) was independent of medium Ca^{2+}, whereas the sustained response was dependent on medium Ca^{2+}. This in turn indicates that the initial pepsinogen release is caused by the intracellular Ca^{2+} release and that the subsequent sustained pepsinogen release may be caused by the initially evoked Ca^{2+} entry from the extracellular space. The fall of initial pepsinogen release from 10 min after the stimulation in the absence of medium Ca^{2+} might reflect both the increase of pepsinogen synthesis and the cessation of pepsinogen

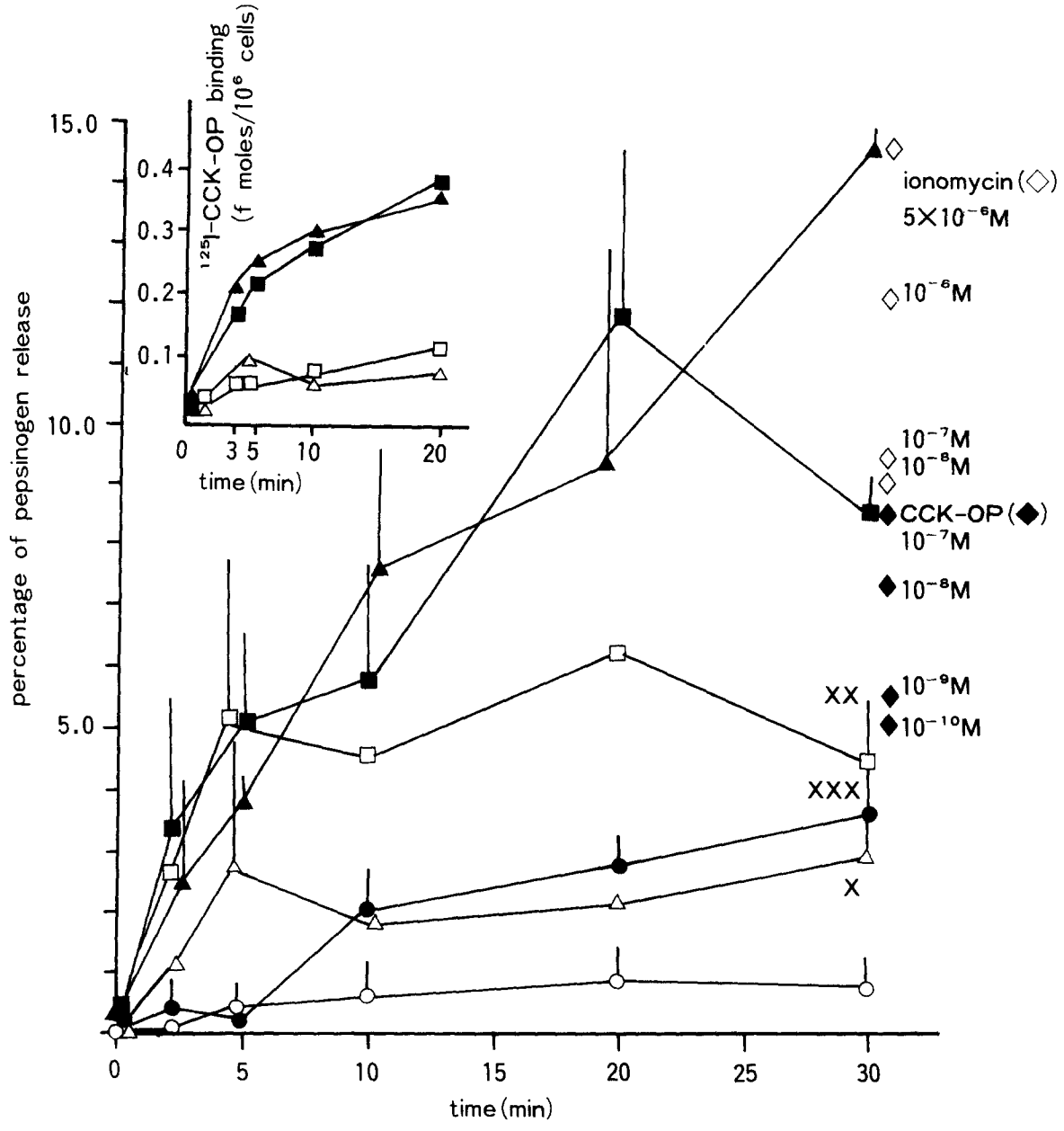

Fig. 5. Time-course of pepsinogen secretion stimulated of gastric glands with CCK-OP and ionomycin. The concentrations of reagents used were as follows: 0.1 μM CCK-OP in the presence (■) or absence (□) of medium Ca^{2+}; 5 μM ionomycin in the presence (▲) or absence (△) of medium Ca^{2+}; control (non-stimulated cells) in the presence (●) or absence (○) of medium Ca^{2+}. The concentration of medium Ca^{2+} was prepared by adding 1.3 mM Ca^{2+} (presence) or 0 mM Ca^{2+} plus 1 mM EGTA (absence). Dose-response curves of CCK-OP (◆) and ionomycin (◇) were determined at 30 min in the presence of medium. X, XX or XXX was the symbol of pepsinogen secretion at 4° C at 30 min that was induced by control, CCK-OP (0.1 μM) and ionomycin (5 μM), respectively. The data represent the mean from seven separate experiments. Pepsinogen secretion at zero-time was subtracted from each value. (Inset) Time-course of ^{125}I-CCK-OP binding to chief cells in the presence or absence of medium Ca^{2+}. Symbol (■) or (▲) was the total binding with and without medium Ca^{2+}, respectively. Symbol (□) or (△) was the nonspecific binding with and without medium Ca^{2+}. The concentration of medium Ca^{2+} was prepared by adding 1.3 mM Ca^{2+} (with) or 0 mM Ca^{2+} plus 1 mM EGTA (without). The data represent the mean from two separate experiments (four determinations). When all of the data were fit by least-squares regression from the data of dose-response curve of isotope-free CCK-OP on specific ^{125}I-CCK-OP binding to chief cells (not shown), the estimated K_d and receptor number per chief cell were $1.604 \cdot 10^{-10}$ M (high affinity) or $1.088 \cdot 10^{-8}$ M (low affinity) and 17500 sites (high affinity) or 281000 sites (low affinity), respectively.

release, since the pepsinogen secretion is expressed as the percentage of total pepsinogen activity present in the cell plus in the medium. The sustained response induced by CCK-OP or ionomycin, which was dependent on medium Ca^{2+}, was dose-dependent and temperature-dependent. Effective concentrations for CCK-OP- or ionomycin-induced changes in pepsinogen secretion at 30 min and initially evoked $[Ca^{2+}]_{cyt}$ in the presence of medium Ca^{2+} were similar in pattern (Fig. 4, inset, right), suggesting a potential role for cellular Ca^{2+} as a mediator of CCK-OP- or ionomycin-induced pepsinogen secretion.

The effects of cytoskeletal-disrupting agents on CCK-OP- or ionomycin-induced initial pepsinogen release

Since the initial Ca^{2+} release evoked by CCK-OP or ionomycin was inhibited by colchicine and cytochalasin D, the effects of these cytoskeletal disrupting agents on CCK-OP- or ionomycin-induced initial and transient pepsinogen release, independent of medium Ca^{2+}, were examined. As shown in Table II, ionomycin or CCK-OP led to a significant release of pepsinogen exceeding that in non-stimulated cells by a factor of 7.88 and 7.38, respectively, at 5 min after the stimulation in the absence of medium Ca^{2+}. These values were little higher than those obtained in Fig. 5, since the pepsinogen release at zero-time was not subtracted. Lanthanum (100 μM) failed to inhibit this pepsinogen release, like Ca^{2+} release, suggesting that the initial but transient pepsinogen release evoked by ionomycin and CCK-OP is mediated by the Ca^{2+} release from the store(s). Pretreatment of cells with colchicine (6 $\mu g/10^6$ cells per 600 μl) or cytochalasin D (6 $\mu g/10^6$ cells per 600 μl) for 10 min at 37°C prior to the stimulation caused an inhibition of ionomycin- or CCK-OP-induced initial pepsinogen release. However, colchicine or cytochalasin D led to a release of pepsinogen in non-stimulated cells exceeding that in non-treated cells with colchicine or cytochalasin D (in the absence of secretagogue) by a factor of 2.15 and 2.71, respectively. Therefore, the statistical analysis was applied again to each control.

Though colchicine and cytochalasin D caused pepsinogen release in the resting state, they significantly inhibited the CCK-OP- or ionomycin-induced initial pepsinogen release. This result suggests that the assembly of the microtubular-microfilamentous system of the chief cell might be involved in CCK-OP- or ionomycin-induced initial pepsinogen release.

TABLE II

THE EFFECTS OF COLCHICINE (COL) AND CYTOCHALASIN D(CD) ON CCK-OP- OR IONOMYCIN-INDUCED INITIAL PEPSINOGEN RELEASE IN THE ABSENCE OF MEDIUM Ca^{2+}

The concentrations of reagents were as follows: 10 μg/ml colchicine; 10 μg/ml cytochalasin D; 100 μM lanthanum (La^{3+}); 10 nM CCK-OP; 5 μM ionomycin. Pepsinogen release with the indicated agents was determined after 5 min incubation in the absence of medium Ca^{2+} (0 mM Ca^{2+} plus 1 mM EGTA). Cell suspension was pre-incubated for 10 min at 37°C with or without colchicine, cytochalasin D and La^{3+} before the cell stimulation. 100% corresponds to each control (control, control + COL, control + CD and control + La^{3+} in non-stimulated cells). The data represent the means ± S.E. from two to four separate experiments.

stimulant	pepsinogen release (percentage of total)	percentage of control (a,b,c,d)	n
control	1.018±0.306 *	100%a)	10
control + COL	2.191±0.264 *	100%b)	9
control + CD	2.756±0.167	100%c)	8
control + La^{3+}	0.993	100%d)	2
ionomycin	8.032±1.658** *	788%v.s.a) *	10
ionomycin + COL	4.031±0.647	184%v.s.b) *	8
ionomycin + CD	7.630±0.215	277%v.s.c) *	8
ionomycin + La^{3+}	8.301	836%v.s.d)	2
CCK-OP	7.511±1.934** *	738%v.s.a) *	9
CCK-OP + COL	5.903±0.928	269%v.s.b) *	9
CCK-OP +CD	6.283±0.382	228%v.s.c) *	8
CCK-OP + La^{3+}	6.132	618%v.s.d)	2

Discussion

Initial Ca^{2+} flux and pepsinogen release

The present study indicates that the IP_3-sensitive and ATP-dependent Ca^{2+} pool is located in or near the endoplasmic reticulum of the guinea-pig chief cell. This concept is supported by the following facts.

(1) There exists a CCCP-, saponin- or digitonin-insensitive but oxalate-sensitive ATP-dependent Ca^{2+} pool in endoplasmic reticulum vesicles.

(2) The quantity of the ATP-dependent Ca^{2+}

taken up by endoplasmic reticulum vesicles in the presence or absence of oxalate in cell-free system was very similar to that taken by plasmalemmal permeabilized and mitochondrial poisoned chief cell.

(3) IP_3 caused substantial Ca^{2+} release from endoplasmic reticulum vesicles that was enhanced by ATP, in both the cell-free and the permeable system as well as the Ca^{2+} ionophore. Permeabilization by 50 μg of saponin per 10^6 chief cells (4.5 mg protein) per ml for 20 min at 37 °C might be appropriate, since pepsinogen leakage and inhibition of ^{125}I-CCK-OP binding to its receptors were obviated under the same experimental condition. Since saponin-permeabilization was accomplished by the concentration of 75 μg/7 mg protein per ml in hepatocytes [22] and 75 μg/7.9 mg protein per ml in parietal cells [36,37], these results conclude that about 10 μg of saponin per 1 mg protein per ml appears to be suited to test the cell function in a permeable system. On the other hand, there exists a high- or low-affinity CCK-OP receptor in the chief cell where $K_d = 1.604 \cdot 10^{-10}$ M, binding sites = 17 500 (high affinity) and $K_d = 1.088 \cdot 10^{-8}$ M, binding sites = 281 000 (low affinity), respectively (see, Fig. 5, legend). The above-described permeable chief cells retained the ability to react to CCK-OP with Ca^{2+} release and the following Ca^{2+} re-uptake without destroying CCK-OP receptors. The sum of the Ca^{2+} released by CCK-OP and IP_3 was constant. The constant but non-additive effect on Ca^{2+} release from the store(s) induced by the combination with CCK-OP and IP_3 indicates that the CCK-OP-induced Ca^{2+} release occurs at the IP_3-sensitive Ca^{2+} pool, since if the CCK-OP-induced Ca^{2+} release and IP_3-promoted Ca^{2+} release occur via different mechanisms, a further Ca^{2+} release by CCK-OP plus IP_3 rather than IP_3 alone should be observed. This in turn suggests that the Ca^{2+} release from the intracellular store(s) (endoplasmic reticulum vesicles) evoked by CCK-OP is mediated by IP_3. Chew and Brown [9] suggested that the Ca^{2+} release in response to CCK-OP and carbachol in rabbit chief cells appears to be mediated by IP_3. The Ca^{2+} re-uptake into the intracellular pool(s) by CCK-OP and IP_3 in the presence of an ATP-regenerating system might be caused by consequent activation of Ca^{2+} pump due to intracellular Ca^{2+} release and subsequent increase in $[Ca^{2+}]_{cyt}$ [36,37]. Thus Ca^{2+} re-uptake also indicates that the pool(s) which release(s) (by IP_3 and CCK-OP) and take(s) up (by ATP) Ca^{2+} is the same or similar. However, Ca^{2+} released by the Ca^{2+} ionophore was not taken up again into the pool(s) under permeabilized circumstances. This may be due to an increase in ATP hydrolysis, because Ca^{2+} recycling across the endoplasmic reticulum membrane by the Ca^{2+} ionophore allows to ATPase to maintain a higher rate of ATP hydrolysis, since no Ca^{2+} gradient is formed [36,37]. The Ca^{2+} release from intracellular store(s) in response to CCK-OP and ionophore was also observed in intact chief cells, as measured by fura-2 acetoxymethyl ester, since the chelation of medium Ca^{2+} by EGTA and the addition of Ca^{2+} entry blocker lanthanum failed to inhibit the increase in $[Ca^{2+}]_{cyt}$ evoked by these ligands. At the same time, the Ca^{2+} entry from the extracellular space was evoked by the stimulation with CCK-OP and Ca^{2+} ionophore exceeding that in Ca^{2+} release by a factor of 2.70–2.75. The CCK-OP-induced Ca^{2+} entry from the medium was almost inhibited by lanthanum. In contrast, it did not always follow that the Ca^{2+} ionophore-induced Ca^{2+} entry was completely inhibited by lanthanum, thus suggesting that the liberation of innermembrane-bound Ca^{2+} (by ionomycin) occurs apart from the entry of external Ca^{2+}, since the chlorotetracycline signal, which reflects inner-membrane-bound Ca^{2+} [45], declined upon ionomycin stimulation. Therefore, the IP_3- and/or CCK-OP-sensitive Ca^{2+} pool is a subset of the Ca^{2+} ionophore-sensitive Ca^{2+} pool. The biphasic pepsinogen secretion from the chief cell evoked by CCK-OP and ionomycin might reflect two types of Ca^{2+} mobilization inasmuch as the initial but transient pepsinogen release was independent of medium Ca^{2+}, due to intracellular Ca^{2+} release, whereas the sustained pepsinogen release was dependent on medium Ca^{2+}, perhaps due to initially evoked Ca^{2+} entry. However, since long exposure to Ca^{2+} chelating agents such as EGTA and EDTA may alter the intracellular Ca^{2+} distribution [38], the exact relationship between Ca^{2+} fluxes and sustained pepsinogen secretion has not yet been substantiated. Since the phorbol ester, TPA(12-*O*-tetradecanoylphorbol 13-acetate), caused a lag period of pepsinogen release followed

by an increased rate of response, Muallem et al. [8] suggested that the sustained pepsinogen secretion is probably mediated by diacylglycerol as opposed to initial pepsinogen release, which may be mediated by IP_3. This study implicates IP_3 as a second messenger for the CCK-OP-stimulated release of Ca^{2+} from the endoplasmic reticulum and subsequent pepsinogen release from the chief cell. The endoplasmic reticulum obtained also contains granular endoplasmic reticulum, zymogen granules and Golgi complex which are candidates for the IP_3-sensitive Ca^{2+} pool. Similar biphasic Ca^{2+} metabolism and secretion were observed with gastrin-stimulated parietal cells in a way evoked by either Ca^{2+} release from an IP_3-sensitive Ca^{2+} pool located in the apical surface and linked with microfilaments or Ca^{2+} entry from the medium, which corresponds to initial acid secretion and the subsequent sustained response, respectively [11,12,36,37].

A role for cytoskeleton on initial Ca^{2+} flux and pepsinogen release

Intracellular Ca^{2+} release evoked by either IP_3, Ca^{2+} ionophores or CCK-OP, Ca^{2+} removal by ATP and CCK-OP- or ionomycin-induced initial pepsinogen release, all of which are associated with stimulus-secretion coupling, were inhibited by the microtubular-microfilamentous disrupting agents, colchicine and cytochalasin D. The inhibitory effects of colchicine and cytochalasin D on intracellular Ca^{2+} metabolism and initial pepsinogen release suggest that the assembly of the microtubular-microfilamentous system might be involved in Ca^{2+}-releasing and -removing mechanism. It has been suggested that colchicine inhibited the biosynthesis of phosphatidylinositol [46]. This might account for the inhibition of the CCK-OP-induced Ca^{2+} release from the store(s) that was induced by colchicine. However, the IP_3-mediated Ca^{2+} release from the store(s) and the subsequent ATP-promoted Ca^{2+} reuptake into the store(s) were also inhibited by colchicine and cytochalasin D, even when IP_3 or ATP was added to the cell suspension under plasmalemmal permeabilized circumstances. Therefore, it is unlikely that the inhibition of the IP_3-mediated Ca^{2+} release from the store(s) induced by colchicine is caused by the inhibition of biosynthesis of phosphatidylinositol. Colchicine and cytochalasin D failed to inhibit the IP_3-mediated Ca^{2+} release from endoplasmic reticulum vesicles in cell-free system. This in turn suggests that the translocation or migration of the IP_3-sensitive Ca^{2+} pool into the lumen in the apical portion by regulation of the microtubular-microfilamentous system after cell stimulation is a prerequisite for causing the Ca^{2+} release and the subsequent exocytosis, since the colchicine or cytochalasin D effect requires some cytosolic elements. It is unlikely that the the observed effects of colchicine and cytochalasin D are due to their cytotoxic effects, since the initial but transient stimulation of basal pepsinogen release evoked by colchicine and cytochalasin D was observed in resting preparations in the absence of secretagogue, whereas the CCK-OP- or Ca^{2+} ionophore-induced pepsinogen release was inhibited by colchicine and cytochalasin D. In addition, colchicine or cytochalasin D did not affect the binding of ^{125}I-CCK-OP to its receptor (not shown). The appropriate concentrations of colchicine ($2.50 \cdot 10^{-5}$ M) and cytochalasin D ($1.97 \cdot 10^{-5}$ M) used in this study were reported by pancreatic β-cells [39], and gastric parietal cells [11,12,32,40]. The mechanism of the slight but significant increase in basal pepsinogen release evoked by colchicine and cytochalasin D in the resting state has not been substantiated; however, a similar effect of colchicine-stimulated transient basal acid secretion from bullfrog gastric mucosa was reported [11].

In conclusion, although the mechanism of the activation of the microtubular-microfilamentous system preceding the Ca^{2+} release has not been substantiated, this study denotes that there is a possibility that CCK-OP and Ca^{2+} ionophores increase the $[Ca^{2+}]_{cyt}$ due to internal Ca^{2+} release from an IP_3-sensitive and ATP-dependent Ca^{2+} pool located in the endoplasmic reticulum at the onset of initial pepsinogen release and that the Ca^{2+} release and concomitant initial pepsinogen release are regulated by the microtubular-microfilamentous system of the chief cell. Gill et al. [41] suggest that in the saponin-permeabilized neuroblastoma cell line, Ca^{2+} accumulated by a store, presumed to be the endoplasmic reticulum, can be released by GTP hydrolysis.

It is also possible that the IP_3- or GTP-depen-

dent system for releasing Ca^{2+} may be closely related, since the IP_3-induced Ca^{2+} release is stimulated by GTP [42]. On the other hand, cytoskeletal assembly is dependent on GTP hydrolysis [43]. This study reveals that the cytoskeletal assembly regulates CCK-OP- or IP_3-induced Ca^{2+} release and initial pepsinogen release. Therefore, it seems that there is a close relationship between GTP hydrolysis, cytoskeletal assembly and hormone-sensitive but IP_3-induced Ca^{2+} release after cell stimulation. Sklar et al. [44] suggest that in human neutrophils, the transient polymerization of actin that is independent of $[Ca^{2+}]_{cyt}$ changes (corresponding to the first phase of right-angle light-scatter response) is followed by a sustained polymerization of actin that requires an increase in $[Ca^{2+}]_{cyt}$ (second phase of response). The assembly of the microtubular-microfilamentous system by the stimulation of cells with hormones may be an early event to cause IP_3-induced Ca^{2+} release and hormone-sensitive biological response. Further investigations will be necessary concerning the mechanism of the cytoskeletal-regulated and medium Ca^{2+}-requiring sustained pepsinogen secretion.

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