Calcium Oscillations in Single Cultured Chinese Hamster Ovary Cells Stably Transfected with a Cloned Human Cholecystokinin $(CCK)_B$ Receptor

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ABSTRACT—In Chinese hamster ovary cells stably expressing the cloned human cholecystokinin $(CCK)_B$ /gastrin receptor, cholecystokinin octapeptide (CCK-8) evoked increases in $[Ca^{2+}]_i$ monitored by digitized video imaging of fura-2 fluorescence ratios. At concentrations around 10 pM, CCK-8 elicited $[Ca^{2+}]_i$ oscillations, which were blocked by elimination of extracellular Ca^{2+}, by a phospholipase C inhibitor, U-73122, by a protein kinase C inhibitor, H7, as well as by phospholipase A_2 (PLA_2) inhibitors, ONO-RS-082 and aristolochic acid. At higher concentrations, CCK-8 induced a single biphasic $[Ca^{2+}]_i$ rise consisting of a large peak followed by a lower sustained plateau, while the response turned into $[Ca^{2+}]_i$ oscillation when the extracellular Ca^{2+} was eliminated or a PLA_2 inhibitor was included. CCK-8 stimulated the release of arachidonic acid, and this was inhibited by aristolochic acid. Arachidonic acid caused an increase in $[Ca^{2+}]_i$ which was dependent upon extracellular Ca^{2+}. These results suggest that the activation of PLA_2 might be involved, at least in part, in the Ca^{2+} influx that maintains the sustained plateau phase of $[Ca^{2+}]_i$ as well as the $[Ca^{2+}]_i$ oscillation when CCK_B receptors are stimulated.

Keywords: CCK_B/gastrin receptor, $[Ca^{2+}]_i$ oscillation, Fura-2, Protein kinase C, Phospholipase A_2

Cholecystokinin (CCK) and gastrin have important physiological roles in the peripheral gastrointestinal system and in the central nervous system as hormones and neurotransmitters (1). CCK receptors have been cloned and classified as CCK_A and CCK_B/gastrin, and subsequently the molecular basis of pharmacological and molecular classification of the receptors is now established. As to the intracellular signal transduction, CCK_A receptor has been extensively studied in isolated pancreatic acinar cells. The CCK_A signal appears to be mediated by the activation of pertussis toxin-insensitive GTP-binding protein(s) and subsequent coupling to phospholipase C (PLC), namely, the activation of protein kinase C (PKC) and the formation of inositol 1,4,5-trisphosphate (IP_3). Using isolated pancreatic acinar cells and CCK_A receptor transfected cells, physiological concentrations of CCK have been shown to elicit a repetitive elevation of $[Ca^{2-}]_i$ or $[Ca^{2+}]_i$ oscillation (2-4), which has been investigated in many different cell types. The signal transduction cascade for the CCK_B receptor has been less well studied than that for the CCK_A receptor, especially because of the experimental difficulties in using native cells expressing CCK_B receptors. Isolation of pure neurons with CCK_B receptors is almost impossible at present. For the secretion of gastric acid, the role of CCK_B receptor on the parietal cell appears to be much less significant than the recently expounded role of CCK_B receptors on enterochromaffin-like cell (5). Using isolated parietal cells or gastric glands, it has been postulated that the cascade after CCK_B receptor activation might be similar to that of CCK_A (1). However, as there is still some possibility for the existence of multiple gastrin receptors other than CCK_B (1), it might be necessary to analyze the signal transduction using a system in which a single class of CCK_B receptors is present. Recently, transfected cells with CCK_B receptors have been available (6, 7). For the $[Ca^{2+}]_i$ oscillation via the CCK_B receptor, however, only one report with isolated canine parietal cells has been published (8), and its mechanism has not yet been elucidated yet. In the present study, we developed a system of Chinese hamster ovary (CHO) cells stably expressing CCK_B receptors as a model to study the mechanism of $[Ca^{2+}]_i$ oscillation in response to physiological concentrations of CCK.

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MATERIALS AND METHODS

Isolation of rabbit gastric glands and dye loading

Rabbit gastric glands were isolated according to Berglindh and Öblink (9). For $[Ca^{2+}]_i$ measurements, a suspension of isolated gastric glands was loaded with 1 μM fura-2/AM in the medium [132.4 mM NaCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 25 mM HEPES-Na (pH=7.4), 11.1 mM glucose, and 1 mg/ml bovine serum albumin] for 30 min at 37℃.

Expression of human CCK_B/gastrin receptor in CHO cells

A stable transformed CHO cell line was established as follows: The full-length cDNA encoding the human CCK_B receptor was kindly provided by Dr. T. Horiuchi (Daiichi Pharmaceutical Co., Ltd., Tokyo). The CCK_B receptor cDNA was subcloned into the Hind III and EcoR I sites of the mammalian expression vector, pUC119. In this expression vector, transcription of the CCK_B receptor was driven by a SV40 early promoter. Resistance to blasticidin S was provided by the cotransfection with pSV2bsr (Kaken Pharmaceutical Co., Ltd., Tokyo), a plasmid of the blasticidin S deaminase encoding gene under the control of the SV40 early promoter. The expression plasmid DNA (2 μg) and Lipofectin (15 μl) were incubated in 200 μl of serum-free growth medium (F-12) for 30 min at 37℃ and diluted with 2 ml of F-12. The mixture was then added to CHO cells ($1-2\times10^5$ cells) cultured on a 60-mm dish. After 18 hr of transfection, the medium was replaced with F-12 medium containing 10% fetal bovine serum. CHO cell clones were established by the selection with 5 μg/ml of blasticidin S.

Radioligand binding assay

CHO cells permanently expressing the cDNA were washed with ice-cold PBS and suspended with lysis buffer [10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA]. The suspension was centrifuged at $30,000\times g$ for 30 min at 4℃. The pellet was resuspended in the lysis buffer and used as the crude membrane fraction. Radioligand binding studies were carried out for 60 min at 25℃ in 1 ml binding buffer [50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$] containing 0.2% bacitracin. The binding reaction was started by the addition of 1 nM of [^{3}H]pBC-264, a selective CCK_B agonist, to the crude membrane and terminated by rapid filtration on glass fiber filters (Whatman GF/C) using a cell harvester. The filter was washed with ice-cold binding buffer containing 0.01% bovine serum albumin, and the radioactivity on the filter was counted with a liquid scintillation counter. Nonspecific binding was determined in the presence of 1 μM of CCK-8.

Measurement of $[Ca^{2+}]_i$ mobilization

$[Ca^{2+}]_i$ in the cells permanently expressing human CCK_B/gastrin receptor was measured by fura-2 fluorometry. Cells cultured on a 15-mm diameter cover glass were loaded with 5 μM fura-2/AM for 30 min at 37℃. After incubation, the cells were washed with a HEPES-buffered salt solution [HBSS; 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1.28 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 10 mM HEPES-Na (pH=7.4), 2 mM L-glutamine, and 5.5 mM glucose]. The cover glass was positioned to a temperature-controlled chamber with a glass cover slip bottom, mounted on the stage of a Nikon Diaphot 300, and continuously superfused at 1 ml/min with HBSS at 37℃. Changes in $[Ca^{2+}]_i$ were measured using the dual-wavelength excitation ratio technique (340/380 nm excitation, 510 nm emission) by a digital imaging system, Argus 50 (Hamamatsu Photonics, Hamamatsu).

Measurement of [^{3}H]arachidonic acid release

Cells permanently expressing human CCK_B/gastrin receptor were incubated with [^{3}H]arachidonic acid (1 μCi/well) for 18–24 hr in a 24-well dish. After washing twice with HBSS, the cells were incubated with various concentrations of CCK-8 for 60 min. Released [^{3}H]-arachidonic acid was quantified with a liquid scintillation counter. When the effect of an inhibitor on the release was to be examined, the cells were preincubated with appropriate concentrations of the inhibitor in HBSS for 60 min at 37℃, after which the medium was aspirated and replaced with a fresh HBSS containing CCK-8 (10 nM) in addition to the inhibitor.

Drugs

Sulfated cholecystokinin octapeptide (CCK-8) and human gastrin were purchased from Peptide Institute (Osaka). Fura-2/acetoxymethylester (Fura-2/AM) was from Dojin Chemicals (Kumamoto). Lipofectin was from Gibco (Grand Island, NY, USA). [^{3}H]pBC-264 (2164.5 GBq/mmol) and [^{3}H]arachidonic acid were from Du Pont (Boston, MA, USA). YM-022 was a gift of Yamanouchi Pharmaceutical Co., Ltd. (Tokyo). ONO-RS-082 was a gift of Ono Pharmaceutical Co., Ltd. (Osaka). U-73122 was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA) via Funakoshi (Tokyo). According to the manufacturer's manual, U-73122 was dissolved in chloroform, dispensed in aliquots and then the solvent was evaporated by a stream of nitrogen, in order to avoid possible inactivation during the storage as a solution. Immediately before use, it was dissolved in dimethylsulfoxide (DMSO). Other chemicals used were analytical grade and purchased from Sigma Chemical (St. Louis, MO, USA).

Statistical analyses

Parametric data were expressed as the mean±S.E. Multiple comparisons were analyzed by ANOVA and Dunnett's post hoc test by a computer program (Super ANOVA; Abacus Concepts, Berkeley, CA, USA). The level of significance was uniformly set at $P<0.05$, and no further calculation of P value was performed.

RESULTS

Selection and characterization of CHO cells transfected with CCK_B receptor

We transfected human CCK_B receptor into CHO cells, and blasticidin S-resistant colonies were screened for functional CCK_B receptors based on [^{3}H]pBC264-bind-

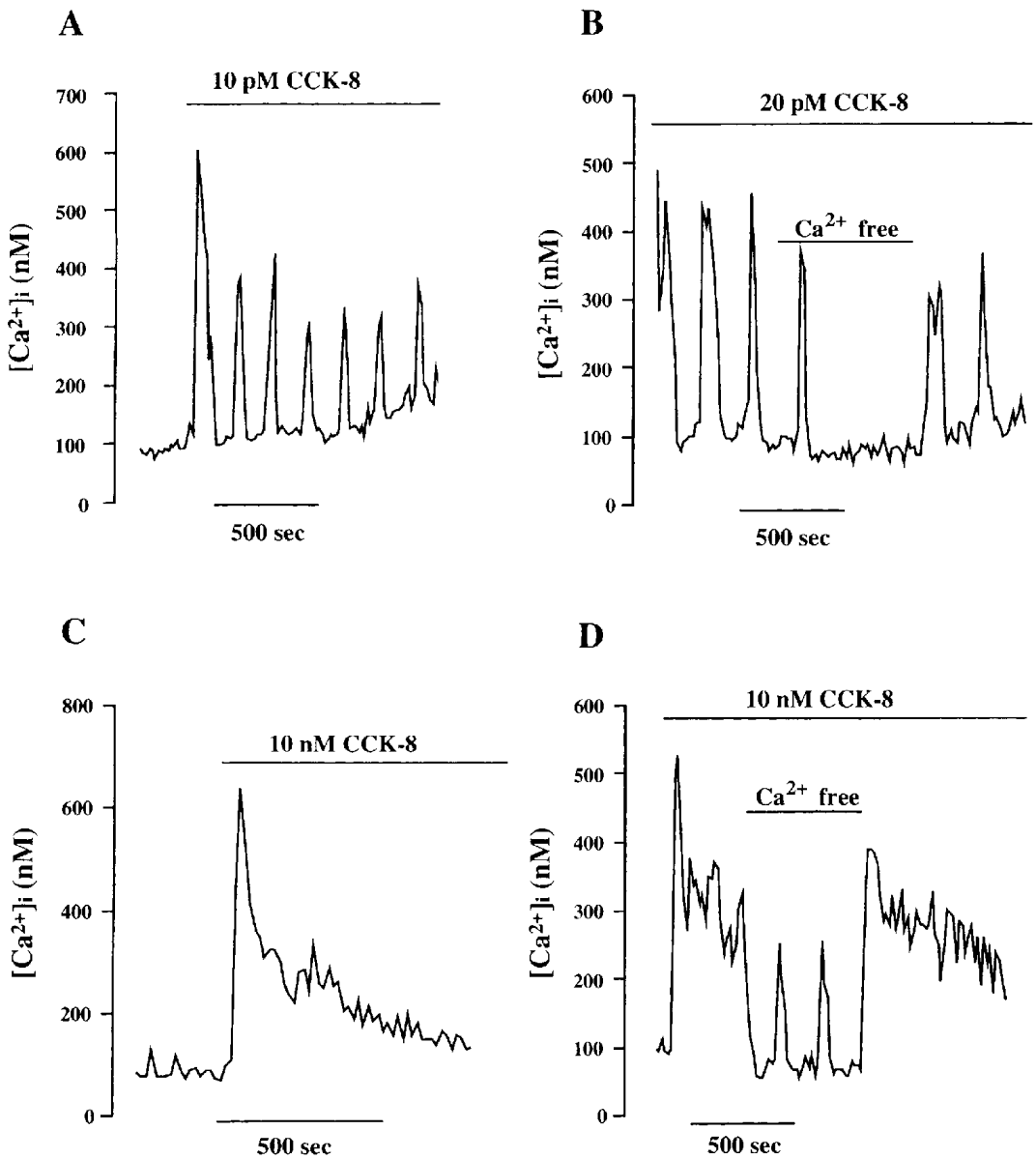

Fig. 1. Cholecystokinin octapeptide (CCK-8)-stimulated intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling in Chinese hamster ovary (CHO) cells expressing CCK_B receptor. A: CCK-8-induced oscillatory pattern in $[Ca^{2+}]_i$ mobilization at low (10 pM) concentration. B: Effect of the removal of the extracellular Ca^{2+} on the response to CCK-8 (20 pM). Note that a complete inhibition, except for the first peak, just after the removal of Ca^{2+}, was obtained. $[Ca^{2+}]_i$ oscillation restarted when Ca^{2+} returned to the normal level. C: CCK-8-induced biphasic rise in $[Ca^{2+}]_i$ at high concentration (10 nM). D: Effect of the removal of the extracellular Ca^{2+} on the response to CCK-8 (10 nM). Note that the sustained plateau was inhibited but turned to $[Ca^{2+}]_i$ oscillation, and the plateau appeared again when Ca^{2+} returned to the normal level. Each plot is a representative of at least 3 experiments where >10 cells were imaged with essentially identical results.

ing. The clone that stably expressed the highest CCK_B receptor was selected from the colonies. Scatchard analysis revealed a single receptor type with a dissociation constant for the ligand of 28.4 nM and a maximal binding capacity of 299 fmol/mg protein. We confirmed that the wild type of CHO cell showed neither the specific binding of [^{3}H]pBC264 nor the $[Ca^{2+}]_i$ response to CCK-8 or gastrin. The following experiments were performed using this clone (CHO-CCK-B cell).

CCK-8 induced $[Ca^{2+}]_i$ signaling in CHO-CCK-B cells

Although it has been reported that CCK and gastrin are not equally acting on the CCK_B receptors (10), in our preliminary experiments using CHO-CCK-B cells loaded with fura-2, both gastrin and CCK-8 showed indistinguishable effects. We thus concluded that the responses by these two agonists were mediated by the same or quite similar pathways, at least with respect to $[Ca^{2+}]_i$ signaling, and we mainly used CCK-8 throughout the study. Superfusion of CCK-8 at concentrations as low as 10 pM elicited a $[Ca^{2+}]_i$ oscillation (Fig. 1A). At this threshold concentration, 73.4% of the cells responded with an average interval of 232.8±19.4 sec and an average $[Ca^{2+}]_i$ concentration of 297.8±26.3 nM above the resting level (mean±S.E. of 35 cells from 3 different experiments). Although 10 pM of CCK-8 was usually enough to elicit $[Ca^{2+}]_i$ oscillation, it was sometimes necessary to increase the concentration up to 20 or 30 pM before oscillatory responses emerged. Thus, the experiments for examining the mechanism of oscillation were performed using 10 to 30 pM of CCK-8, depending upon variations in the condition of the cell.

The oscillatory response remained constant in amplitude and frequency for up to 30 min. Further observation tended to be difficult because of the attenuation of fluorescence intensity due to the loss of dye. Elimination of extracellular Ca^{2+} during $[Ca^{2+}]_i$ oscillation abolished the repetitive Ca^{2+} spikes without affecting the first peak, and this inhibition was reversed by the re-addition of Ca^{2+} to the extracellular medium (Fig. 1B).

At concentrations higher than 1 nM, CCK-8 caused a transient rise in $[Ca^{2+}]_i$ with a duration of 2–5 min. This peak of $[Ca^{2+}]_i$ was followed by a slower decrease to a much lower but still elevated plateau (Fig. 1C). As shown in Fig. 1D, elimination of the extracellular Ca^{2+} rapidly reversed the sustained elevation of $[Ca^{2+}]_i$ to the resting level and converted it into $[Ca^{2+}]_i$ oscillation. In Ca^{2+}-free medium, each transient rise of $[Ca^{2+}]_i$ was not accompanied by the plateau phase.

Effects of various inhibitors on CCK-induced $[Ca^{2+}]_i$ signaling

As expected, $[Ca^{2+}]_i$ responses elicited by CCK-8 were completely blocked by YM022 (10 nM), a CCK_B/gastrin receptor specific antagonist (11), confirming that the responses were mediated by the CCK_B receptor (data not shown). In CHO-CCK-B cells exhibiting $[Ca^{2+}]_i$ oscillation by 10 pM of CCK-8, concurrent perfusion of U-73122 (3 μM), a PLC inhibitor, abolished the $[Ca^{2+}]_i$ response (Fig. 2). The $[Ca^{2+}]_i$ increase induced by CCK-8 was not affected by pretreatment of the cell with pertussis toxin (100 ng/ml for 18–24 hr), excluding the possible involvement of Gi in the signal transduction.

Role of protein kinase C and phospholipase A_2 in $[Ca^{2+}]_i$ oscillation

In order to assess the possible role of PKC, we first determined how H7, a PKC inhibitor, to affected the $[Ca^{2+}]_i$ change with 10 nM of CCK-8 (Fig. 3: A and B). Under the control condition, the initial transient rise in $[Ca^{2+}]_i$ was 617.3±30.6 nM and the sustained plateau was 121.3±19.4 nM above the resting level at 10 min after the initial peak (mean±S.E. of 20 cells from 3 separate experiments). It is obvious from Fig. 3, A and B, when 10 μM H7 was included, the initial peak was not affected but the sustained plateau was markedly reduced. The same concentration of H7 abolished the $[Ca^{2+}]_i$ oscillation induced by CCK-8 without any changes in basal $[Ca^{2-}]_i$ (data not shown). As these results strongly suggested the possible involvement of PKC-mediated Ca^{2+} influx, we then tested the effects of phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 5 μM), a PKC activator. With this treatment, the frequency of CCK-8-induced oscillation was unaffected, whereas the baseline of

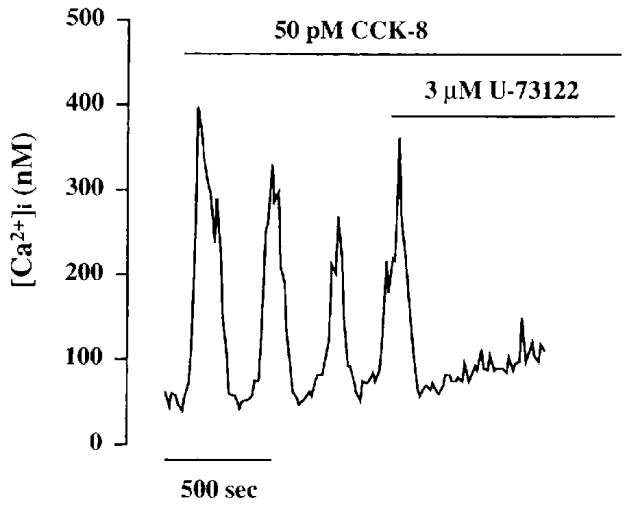

Fig. 2. U-73122 inhibits the $[Ca^{2+}]_i$ oscillation stimulated by CCK-8. In individual cells stimulated with CCK-8 (50 pM), concurrent perfusion with U-73122 (3 μM) rapidly inhibited the $[Ca^{2+}]_i$ signal. The trace is a representative of >10 individual cells imaged, in 3 separate experiments with essentially identical results.

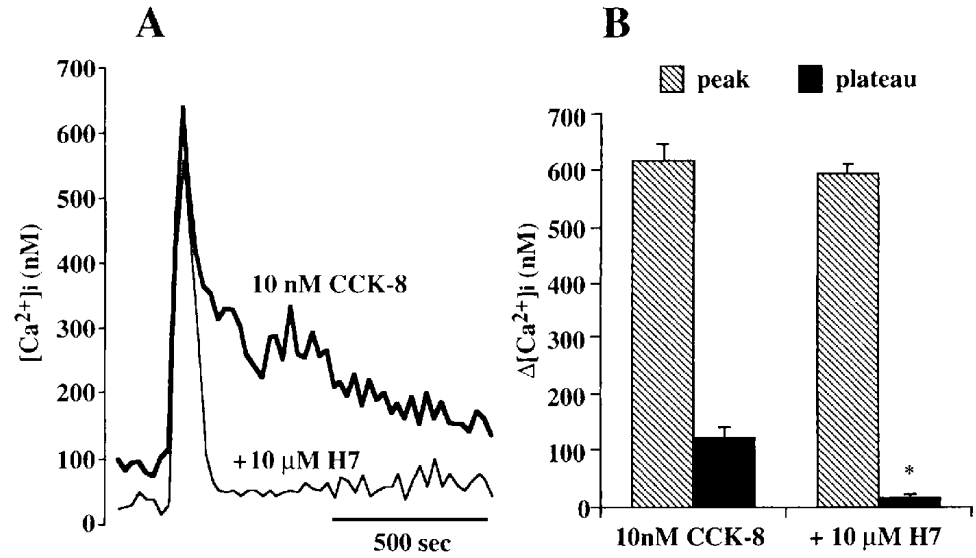

Fig. 3. H7 inhibits the plateau phase, but not the initial phase of the $[Ca^{2-}]_i$ response induced by CCK-8. A: Upper thick trace represents the response to 10 nM CCK-8, and the lower fine trace is the response in the presence of 10 μM H7 added 10 min before. B: The initial transient rise in $[Ca^{2+}]_i$ (peak: hatched column) and the sustained plateau level at 10 min after the initial peak (plateau: filled column) by 10 nM CCK-8 in the absence (left panel) or the presence (right panel) of 10 μM H7 were quantified as $\Delta[Ca^{2+}]_i$ above resting values and expressed as the mean$\pm$S.E. of 20 cells from 3 separate experiments. *Significantly different from the plateau value without H7 at $P<0.05$.

$[Ca^{2+}]_i$ was increased to obscure the oscillatory changes (Fig. 4). To elucidate the role of PKC by itself, the cells were treated with 5 μM of TPA in the absence of CCK-8. TPA caused a sustained increase in $[Ca^{2+}]_i$ to 33.3$\pm$3.7 nM above the basal level (abolished by $[Ca^{2+}]_o=0$). Interestingly, a phospholipase A_2 (PLA_2) inhibitor, ONO-RS-082 at 1 μM, significantly inhibited the $[Ca^{2+}]_i$ increase induced by TPA (3.7$\pm$2.9 nM above basal, 16 cells from 3 separate experiments, $P<0.05$ vs TPA control). Thus involvement of PLA_2 in Ca^{2+} influx was suggested.

We then applied PLA_2 inhibitors on the cells exhibiting $[Ca^{2+}]_i$ oscillation. Aristolochic acid (50 μM) completely abolished $[Ca^{2+}]_i$ oscillation induced by the low concentration of CCK-8 (10 pM, Fig. 5A). However, when CCK-8 was increased to 10 nM, oscillatory changes in $[Ca^{2+}]_i$ were observed (Fig. 5B). Similar observations were also obtained with another PLA_2 inhibitor, ONO-RS-082 (1 μM; Fig. 5, C and D). In contrast, neither indomethacin (30 μM), a cyclooxygenase inhibitor, nor AA861 (10 μM), a lipoxygenase inhibitor, affected $[Ca^{2-}]_i$ responses induced by CCK-8 (data not shown).

Arachidonic acid increases the Ca^{2+} influx

In the previous section, it was suggested that the products of PLA_2, probably arachidonic acid, is involved in the responses via CCK_B receptor, especially in the Ca^{2+} influx. We thus applied arachidonic acid (10 μM) exogenously to CHO cells and found that it caused a sustained increase in $[Ca^{2+}]_i$ (59.2$\pm$6.7 nM above basal, 16 cells from 3 separate experiments). This increase was abolished by the elimination of the extracellular Ca^{2+} (Fig. 6). Indomethacin (30 μM, 10 min before stimula-

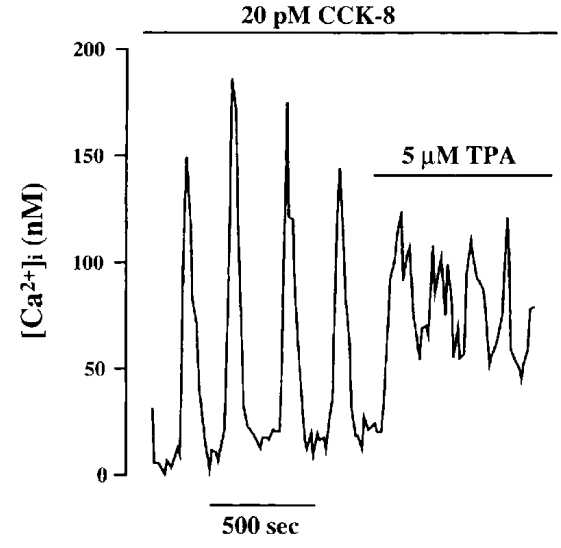

Fig. 4. TPA-induced sustained $[Ca^{2+}]_i$ elevation during CCK-8 stimulated $[Ca^{2+}]_i$ oscillation. In the cell stimulated by CCK-8, concurrent perfusion with TPA (5 μM) increased the basal level of $[Ca^{2+}]_i$, whereas some oscillatory responses still remained. The trace is a representative of >10 cells imaged, in 3 separate experiments with essentially identical results.

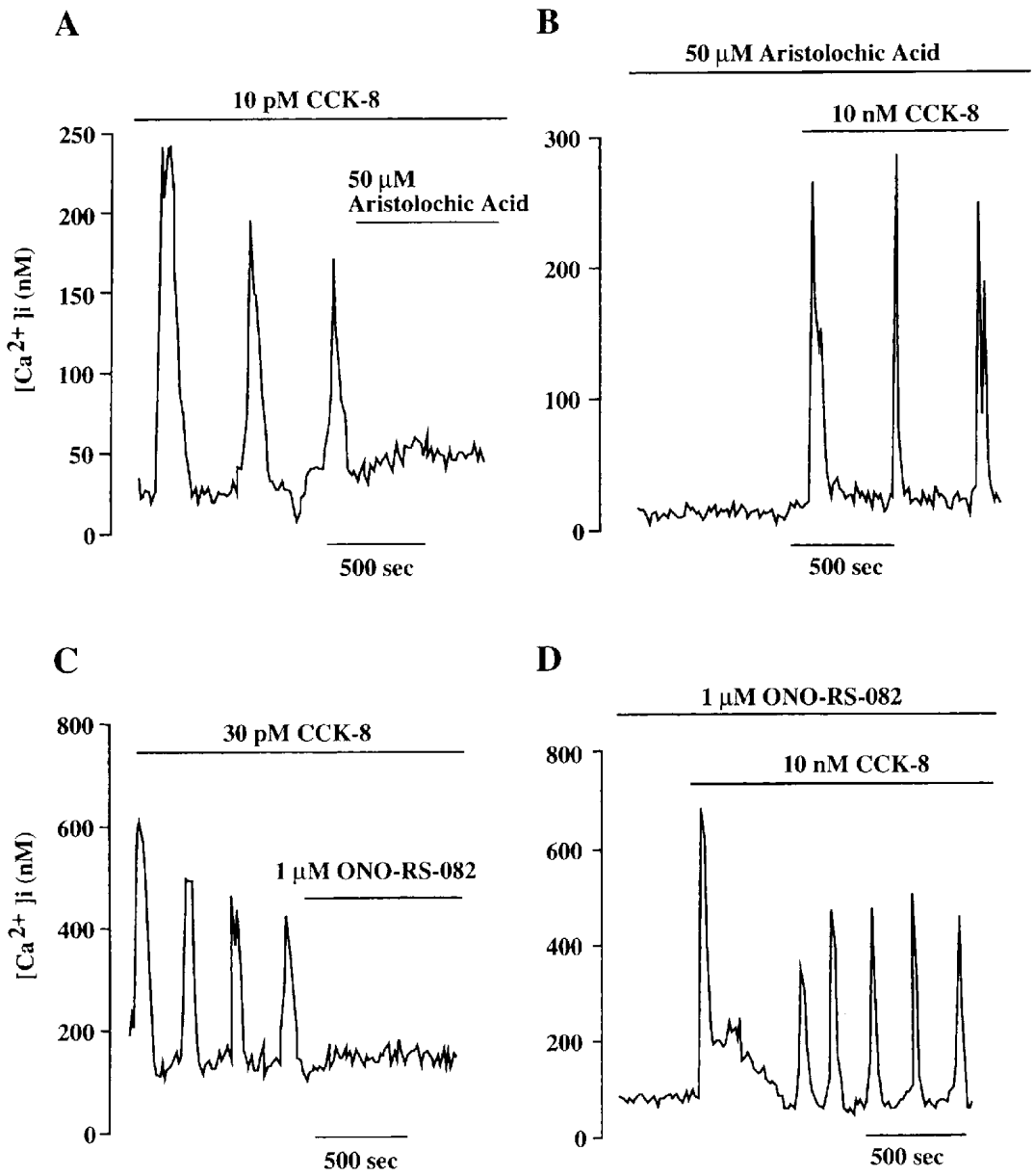

Fig. 5. Effects of aristolochic acid and ONO-RS-082, phospholipase A_2 inhibitors, on $[Ca^{2+}]_i$ signaling stimulated by CCK-8. A: In the cell stimulated by a low concentration of CCK-8 (10 pM), concurrent perfusion with aristolochic acid (50 μM) abolished the $[Ca^{2+}]_i$ oscillation. B: Under the presence of 50 μM aristolochic acid, a high concentration of CCK-8 (10 nM), which usually induces a single, biphasic $[Ca^{2+}]_i$ response, elicited $[Ca^{2+}]_i$ oscillation. C: In the cell stimulated by low concentration of CCK-8 (30 pM), concurrent perfusion with ONO-RS-082 (1 μM) abolished the $[Ca^{2-}]_i$ oscillation. D: Under the presence of 1 μM ONO-RS-082, a high concentration of CCK-8 (10 nM) elicited $[Ca^{2+}]_i$ oscillation as in the case of aristolochic acid. Each trace is a representative of >10 cells imaged, in 3 separate experiments with essentially identical results.

tion) failed to inhibit the arachidonic acid-induced $[Ca^{2+}]_i$ increase (68.2 ± 14.5 nM above basal, 16 cells from 3 separate experiments), and AA861 (10 μM, 10 min before stimulation) had no statistically significant effect on the $[Ca^{2+}]_i$ increase, although there was a tendency to inhibit the increase (39.1 ± 5.5 nM above basal, 16 cells from 3 separate experiments).

Arachidonic acid release via the activation of CCK_B receptor

In order to confirm the activation of PLA_2 by CCK-8, we measured the release of arachidonic acid from CHO-CCK-B cells. As shown in Fig. 7A, CCK-8, from 100 pM to 100 nM, dose-dependently stimulated the release of [^{3}H]arachidonic acid. The increase in arachidonic acid release by 10 nM CCK-8 was dose-dependently inhibited by

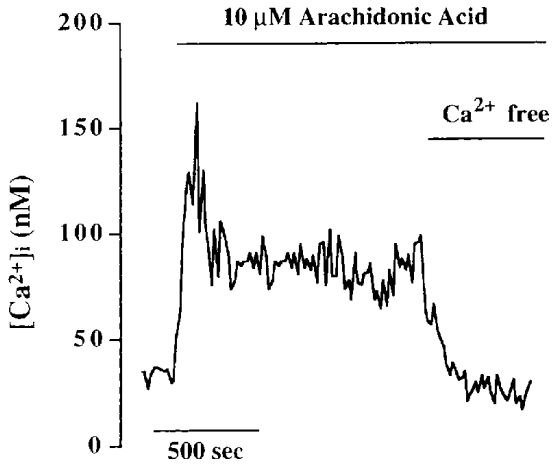

Fig. 6. Arachidonic acid-induced sustained $[Ca^{2+}]_i$ elevation. Individual cells stimulated by arachidonic acid (10 μM) increased $[Ca^{2+}]_i$ to produce a sustained plateau, which rapidly returned to the basal level by the elimination of the extracellular Ca^{2+}. The trace is a representative of >10 cells imaged, in 3 separate experiments with essentially identical results.

the pretreatment with the PLA_2 inhibitor aristolochic acid (Fig. 7B). The maximal inhibition was obtained with 50 μM of the inhibitor and this concentration was used for the $[Ca^{2+}]_i$ measurements.

Gastrin-induced $[Ca^{2+}]_i$ oscillation in isolated rabbit gastric glands

Administration of 100 pM gastrin to isolated rabbit gastric glands loaded with fura-2 elicited $[Ca^{2+}]_i$ oscillation in parietal cells (Fig. 8). At higher concentrations of gastrin, the response turned to a single large peak followed by a much lower but still elevated plateau. These observations suggest that our transfected CHO-CCK-B cells were representative of native cells with CCK_B receptors. However, the responsiveness of the glands to gastrin was poor compared with that to carbachol or histamine, and the oscillatory responses were only rarely observed (e.g., 1 or less out of 8 cases). Therefore, it was almost impossible to perform routine experiments to elucidate the mechanism of $[Ca^{2+}]_i$ oscillation using the native cells expressing CCK_B receptors.

DISCUSSION

There have been a few reports that cells transfected with CCK_B receptors elicited an increase in $[Ca^{2+}]_i$ in response to CCK or gastrin (6, 7), whereas no information has been available about CCK_B receptor-mediated $[Ca^{2+}]_i$ oscillation in transfected cells. In our CHO-CCK-B cells, low concentrations of CCK-8 (about 10 pM) consistently elicited $[Ca^{2+}]_i$ oscillation, while higher concentrations of CCK-8 (about 10 nM) caused a biphasic rise in $[Ca^{2+}]_i$, an initial transient rise followed by a sus-

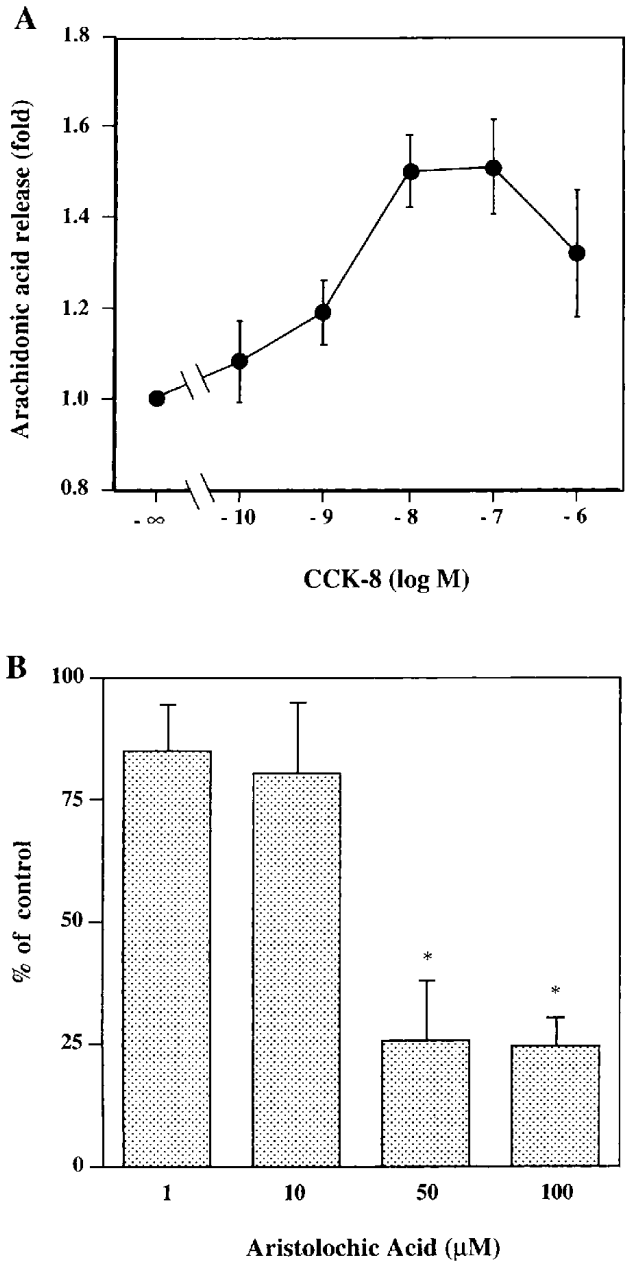

Fig. 7. CCK-8-induced arachidonic acid release and its inhibition by aristolochic acid. A: CHO-CCK-B cells were loaded with [^{3}H]-arachidonic acid for 18–24 hr and then stimulated by CCK-8 for 60 min. Released [^{3}H]arachidonic acid was quantified and expressed as the ratio to the value of the vehicle control. B: Inhibitory effects of aristolochic acid on the CCK-8-induced arachidonic acid release. The cells loaded with [^{3}H]arachidonic acid were preincubated with the inhibitor for 60 min and then stimulated by CCK-8 (10 nM) in the presence of the inhibitor. Released [^{3}H]arachidonic acid was quantified and expressed as % of the increment in CCK-8 control. Data are means±S.E. of 3 experiments performed in duplicate. *$P<0.05$ vs control (Dunnett's test after ANOVA).

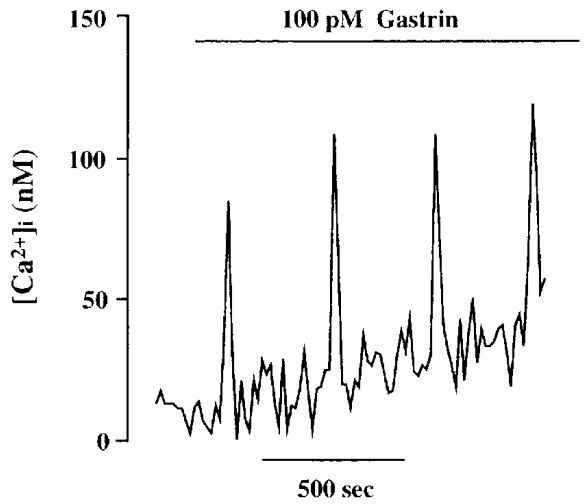

Fig. 8. Gastrin (100 pM)-evoked $[Ca^{2+}]_i$ oscillation in a single rabbit parietal cell. Isolatedrabbit gastric glands were loaded with fura-2, and parietal cell within the glands were identified according to their characteristic morphology. The trace is a representative of 3 cells eliciting $[Ca^{2+}]_i$ oscillation among 25 parietal cells imaged.

tained plateau. As we have encountered some difficulty in analyzing the CCK_B receptor-mediated signaling using native cells, we considered our system as quite a useful model especially for investigating the mechanism of $[Ca^{2+}]_i$ oscillation. The model system also provides an advantage in facilitating the analysis of responses mediated by a single class of cloned CCK_B receptor instead of those mediated by possibly heterologous receptor populations on the native cells (1, 3).

The $[Ca^{2+}]_i$ responses to CCK of CHO-CCK_B cells were resistant to the treatment with pertussis toxin. U-73122, a PLC inhibitor, blocked the $[Ca^{2+}]_i$ oscillation induced by CCK-8. Although the $[Ca^{2+}]_i$ oscillation was susceptive to the removal of extracellular Ca^{2+}, both the first peak of the oscillation and the initial transient rise by high concentrations of CCK-8 were resistant to Ca^{2+} removal. These observations indicate that $[Ca^{2+}]_i$ signaling by CCK-8 is mediated by pertussis toxin-insensitive GTP binding protein-coupled inositol lipid turnover, and as generally accepted, IP_3 is involved in the release of Ca^{2+} from the intracellular store. This is consistent with the observations that stimulation of CCK_B receptor increased the inositol lipid turnover, causing the elevation of $[Ca^{2+}]_i$ in CHO cells expressing CCK_B receptor (6), in rabbit parietal cells (12), and in canine parietal cells (8), although the occurrence of $[Ca^{2-}]_i$ oscillations was only described in the last report.

As to the mechanism of Ca^{2+} oscillation, the emerging consensus seems to be that the basic machinery requires the propagation of two functional activities within the cell: one is the positive feedback whereby Ca^{2+} effects its own release from the interior stores through a process of Ca^{2+}-induced Ca^{2+} release, and the other is the recovery process of refilling the stores (13, 14). In this model, agonist-induced Ca^{2+}-influx is considered to be essential and to play an important role in sensitizing the Ca^{2+}-induced Ca^{2+} release upon refilling the Ca^{2+}-stores. The process involved in the Ca^{2+}-induced Ca^{2+} release (using either the IP_3 or ryanodine receptor) and also the mechanism of Ca^{2-}-influx are both dependent on the type of cells and agonists. In the present observations, Ca^{2-} oscillations induced by the activation of CCK_B receptors on the CHO cell membrane are in line with the mechanisms mentioned above. Namely, the Ca^{2+} oscillation with the low concentration of CCK-8 was terminated by the elimination of Ca^{2-} in the media, indicating that the response was dependent upon the influx of extracellular Ca^{2+}. The role of Ca^{2+}-influx seems to be more important for sensitizing the IP_3-receptor rather than for refilling the Ca^{2+}-stores, since the elimination of extracellular Ca^{2+} caused a conversion of the sustained phase into the oscillatory changes in $[Ca^{2+}]_i$, as the cell was stimulated by a high concentration of CCK-8 and high level of IP_3.

The mechanisms of Ca^{2+} influx in the sustained phase following initial transient rise after agonist stimulation have been extensively studied in a variety of cell types (15). The so-called capacitative calcium entry, activated by the depletion of the Ca^{2+} store, has been hypothesized, and one of the candidate cation channels was recently cloned (16). However, the mechanism for the channel activation is still unknown.

In the present study, it was suggested that PKC might be involved in the Ca^{2+} influx into CHO cells mediated by CCK_B receptors, based on observations that H7, a PKC inhibitor, specifically inhibited the sustained phase of Ca^{2+} entry, but not the initial phase, and that TPA, a PKC activator, produced a sustained increase in $[Ca^{2+}]_i$. Furthermore, it was suggested that PLA_2 might be involved in the Ca^{2+} influx stimulated by CCK_B as well because: a) PLA_2 inhibitors, ONO-RS-082 and aristolochic acid, caused effects similar to that of the elimination of extracellular Ca^{2+} on the CCK_B mediated Ca^{2+} responses, b) arachidonic acid evoked Ca^{2+} influx, and, c) activation of CCK_B receptors stimulated arachidonic acid release from the cell. At present, we do not have any data to judge whether PKC and PLA_2 are activated sequentially or independently. Although it is possible that the activation of PLA_2 was the consequence of $[Ca^{2+}]_i$ elevation, there are reports suggesting the possibility that PKC activates PLA_2 directly (17) or indirectly through MAP kinase activation (18). We are now examining the possibility that the stimulation of CCK_B receptor leads to the activation of PLA_2 directly or indirectly via PKC.

We also examined whether Ca^{2+} influx was induced by arachidonic acid itself or its metabolites. Neither cyclooxygenase inhibitors nor lipoxygenase inhibitors affected arachidonic acid-induced Ca^{2-} influx, suggesting that arachidonic acid itself causes Ca^{2+} influx in CHO-CCK-B cells. Although its precise mechanism of action is presently unknown, arachidonic acid has been implicated as a direct modulator of the Ca^{2+} channel (19, 20).

In the present study, CHO-CCK-B cells showed quite similar properties to the CHO cells expressing CCK_A receptors (3). In the experiments with CCK_A receptors, low concentrations of CCK-8 elicited $[Ca^{2+}]_i$ oscillations that were blocked by U-73122. At higher concentrations, CCK-8 induced a biphasic response and increased the release of arachidonic acid. Therefore, the intracellular signal transduction of the CCK_B receptor appears to be quite similar to that of the CCK_A receptor.

In order to confirm the physiological significance of our observations, we examined the role of CCK_B receptors in the $[Ca^{2+}]_i$ mobilization using native cells, i.e., isolated rabbit gastric glands. We observed that low concentrations of gastrin elicited $[Ca^{2+}]_i$ oscillation in rabbit parietal cells, although the frequency and reproducibility of the observation were low. Similar oscillations induced by 100 pM gastrin were also reported using isolated canine parietal cells (8). In rat pancreatic acinar cells, the $[Ca^{2+}]_i$ oscillation induced by CCK-8 was assumed to be relevant to digestive enzyme secretion (21). In the case of rabbit gastric glands, however, 100 pM of gastrin, which caused $[Ca^{2+}]_i$ oscillation, had no stimulatory effects on acid secretion by itself. Although $[Ca^{2+}]_i$ oscillation does not appear to be relevant to the direct activation of acid secretion, the fact that $[Ca^{2+}]_i$ oscillation was induced by physiological concentrations of gastrin suggests a potential regulatory role of this typical $[Ca^{2+}]_i$ movement in the parietal cell, e.g., the potentiating interaction with other agonists under certain conditions (22, 23).

In summary, the $[Ca^{2-}]_i$ oscillation induced by the weak activation of CCK_B receptor requires Ca^{2+} influx to support the activity of the small amount of IP_3. At least some part of the Ca^{2+} influx might be mediated by the activation of PLA_2 and subsequent arachidonic acid production. When the receptor is fully activated, the Ca^{2+} influx becomes so high that a sustained plateau phase is maintained and $[Ca^{2+}]_i$ oscillation no longer occurs. An important challenge should be to determine the mechanism of CCK_B receptor-mediated events in native cells, such as parietal cells and nerve cells, based on the present study.

Acknowledgments

The authors thank Dr. T. Horiuchi (Daiichi Pharmaceutical Co., Ltd., Tokyo) for the generous gift of the CCK_B receptor clone. Yamanouchi Pharmaceutical Co., Ltd. (Tokyo) and Ono Pharmaceutical Co., Ltd. (Osaka) are also gratefully acknowledged for the supply of YM022 and ONO-RS-082, respectively. The authors thank Dr. John G. Forte (University of California, Berkeley) for critical reading of the manuscript.

REFERENCES

1 Wank SA: Cholecystokinin receptors. Am J Physiol **269**, G628–G646 (1995)
2 Yule DI and Williams JA: U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not JMV-180 in rat pancreatic acini. J Biol Chem **267**, 13830–13835 (1992)
3 Yule DI, Tseng MJ, Williams JA and Logdson CD: A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. Am J Physiol **265**, G999–G1004 (1993)
4 Smith JP, Yelamarty RV, Kramer ST and Cheung JY: Effects of cholecystokinin on cytosolic calcium in pancreatic duct segments and ductal cells. Am J Physiol **264**, G1177–G1183 (1993)
5 Prinz C, Scott DR, Hurwitz D, Helander HF and Sachs G: Gastrin effects on isolated rat enterochromaffin-like cells in primary culture. Am J Physiol **267**, G663–G675 (1994)
6 Ito M, Matsui T, Taniguchi T, Tsukamoto T, Murayama T, Arima N, Nakata H, Chiba T and Chihara K: Functional characterization of a human brain cholecystokinin-B receptor. A trophic effect of cholecystokinin and gastrin. J Biol Chem **268**, 18300–18305 (1993)
7 Seufferlein T, Withers DJ, Broad S, Herget T, Walsh JH and Rozengurt E: The human CCK_B/gastrin receptor transfected into Rat1 fibroblasts mediates activation of MAP kinase, $p74^{raf-1}$ kinase, and mitogenesis. Cell Growth Differ **6**, 383–393 (1995)
8 DelValle J, Tsunoda Y, Williams JA and Yamada T: Regulation of $[Ca^{2+}]_i$ by secretagogue stimulation of canine gastric parietal cells. Am J Physiol **262**, G420–G426 (1992)
9 Berglindh T and Öbrink KJ: A method for preparing isolated glands from rabbit gastric mucosa. Acta Physiol Scand **96**, 150–159 (1976)
10 Lallement J-C, Oiry C, Lima-Leite A-C, Lignon M-F, Fulcrand P, Galleyrand J-C and Martinez J: Cholecystokinin and gastrin are not equally sensitive to GTPγS at CCK_B receptors: importance of the sulphated tyrosine. Eur J Pharmacol **290**, 61–67 (1995)
11 Saita Y, Yazawa H, Honma Y, Nishida A, Miyata K and Honda K: Characterization of YM022, its CCK_B/gastrin receptor binding profile and antagonism to CCK-8-induced Ca^{2+} mobilization. Eur J Pharmacol **269**, 249–254 (1994)
12 Chew C and Brown MR: Release of intracellular Ca^{2+} and elevation of inositol triphosphate by secretagogues in parietal and chief cells isolated from rabbit gastric mucosa. Biochim Biophys Acta **888**, 116–125 (1986)
13 Berridge MJ: Inositol trisphosphate and calcium signalling. Nature **361**, 315–325 (1993)
14 Divecha N and Irvine RF: Phospholipid signaling. Cell **80**, 269–278 (1995)
15 Putney JW and Bird GSJ: The signal for capacitative calcium entry. Cell **75**, 199–201 (1993)
16 Zitt C, Zobel A, Obukhov AG, Hartenek C, Kalbrenner F, Lückhoff A and Schultz G: Cloning and functional expression of a human Ca^{2+}-permeable cation channel activated by calcium store depletion. Neuron **16**, 1189–1196 (1996)

17 Lin L-L, Lin AY and Knopf JL: Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. Proc Natl Acad Sci USA **89**, 6147–6151 (1992)
18 Xing M and Insel PA: Protein kinase C-dependent activation of cytosolic phospholipase A_2 and mitogen-activated protein kinase by alpha1-adrenergic receptors in Madin-Darby canine kidney cells. J Clin Invest **97**, 1302–1310 (1996)
19 Huang J, Xian M-CH and Bacaner M: Long-chain fatty acids activate calcium channels in ventricular myocytes. Proc Natl Acad Sci USA **89**, 6452–6456 (1992)
20 Shimasue K, Urushidani T, Hagiwara M and Nagao T: Effects of anandamide and arachidonic acid on specific binding of (±)-PN200-110, diltiazem and (−)-desmethoxyverapamil to L-type Ca^{2+} channel. Eur J Pharmacol **296**, 347–350 (1996)
21 Zhang BX and Muallem S: Feedback inhibition of Ca^{2+} release by Ca^{2+} is the underlying mechanism of agonist-evoked intracellular Ca^{2+} oscillations in pancreatic acinar cells. J Biol Chem **267**, 24387–24393 (1992)
22 Soll AH: Potentiating interactions of gastric stimulants on [^{14}C]aminopyrine accumulation by isolated canine parietal cells. Gastroenterology **83**, 216–223 (1982)
23 Li Z-Q, Cabero JL and Mårdh S: Gastrin and carbachol require cAMP to elicit aminopyrine accumulation in isolated pig and rat parietal cells. Am J Physiol **268**, G82–G89 (1995)