Signal transduction pathways mediating CCK-induced gallbladder muscle contraction

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CCK is a major physiological hormone that regulates gallbladder contraction and emptying of bile during the intestinal phase of the postprandial state. CCK-induced gallbladder contraction is partly myogenic and induced gallbladder contraction is partly myogenic and mediated by activation of the pertussis toxin (PTx) sensitive G13 protein coupled with PLC-1 (PLC-

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squares were digested in a collagenase solution and gassed with 100% O₂ for 2.5–3 h at 31°C. The collagenase solution contained 150 U of type II collagenase per milliliter of HEPES-buffered nutrient salt solution. The HEPES buffer solution contained (in mM) 112.5 NaCl, 5.5 KCl, 2.0 KH₂PO₄, 24 HEPES, 1.9 CaCl₂, 0.6 MgCl₂, and 10.8 glucose, as well as 0.08 mg/ml soybean trypsin inhibitor and 2% (vol/vol) basal medium Eagle (50:50) amino acids without l-glutamine. The partly digested tissue was then washed over a Nitex mesh with collagenase-free HEPES buffer and incubated in the same buffer for 30–60 min to allow muscle cells to disperse freely. Cells were then harvested by filtering through a Nitex mesh, and the filtrate (cell suspension) was equilibrated for 20 min before the experiment was started. For some experiments, cells were permeabilized with a brief exposure to saponin (75 µg/ml for 4 min) and equilibrated in a modified cytosolic buffer, which consisted of (in mM) 20 NaCl, 100 KCl, 25 NaHCO₃, 5.0 MgSO₄, 0.96 NaH₂PO₄, 0.48 CaCl₂, 1.0 EGTA, 0.01 antimycin A, 1.5 ATP, and an ATP-regenerating system consisting of creatine phosphokinase (10 U/ml) and 5 mM creatine phosphate (48).

Aliquots of 0.25 ml cell suspension were added to the test tubes containing 0.1 ml buffer with appropriate concentrations of agonists or antagonists. Antagonists were preincubated with muscle cells for 60 s before cells were exposed to agonists for 30 s. The response was stopped by the addition of acrolein at a final concentration of 1%. A few drops of the fixed cells were placed on a microscope slide and covered with a coverslip. The lengths of 30 undamaged cells from control and experimental samples were measured with a Zeiss phase-contrast microscope (Carl Zeiss, Oberkochen, Germany), a Panasonic CCTV camera (model WV-CD51; Matsushita Communication Industry, Osaka, Japan), and a Macintosh IIci computer (Apple Computer, Cupertino, CA), using a computer software program, Image 1.33 (National Institutes of Health, Bethesda, MD). Contraction was expressed as percent shortening from initial control length.

Preparation of gallbladder muscle membranes. Gallbladder muscle squares were homogenized with a Tissue Tearor (Biospec, Racine, WI) for three bursts of 20 s each at setting 5 in 20 mM ice-cold HEPES-homogenized buffer (pH 7.4) and again with 60 strokes of a Dounce Grinder (Wheaton, Millville, NJ). The homogenates were centrifuged at 600 g for 2 min. The supernatant was collected and the pellet was rehomogenized and filtered through two layers of 200-µm Nitex mesh. The pooled supernatant was then ultracentrifuged at 40,000 × g for 50 min. The supernatant was removed and the pellet was resuspended in 20 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1% Triton X-100, 2 mM PMSF, 0.1 mM dithiothreitol (DTT), and 2 µg/ml leupeptin and centrifuged at 12,000 × g for 5 min. The supernatant was loaded and subjected to 8% SDS-PAGE (Mini-PROTEAN II cell; Bio-Rad, Hercules, CA). Prestained molecular weight markers were run in an adjacent lane to allow molecular weight to be determined. The separated proteins were electrical transferred at 4°C to a nitrocellulose membrane (Bio-Rad, Melville, NY) in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. The nitrocellulose membranes were blocked with 5% dry nonfat milk blocking 0.2% Tween-20 at room temperature for 1 h, followed by incubation with different anti-PLC-β isoenzyme-specific antibodies (1 µg/ml) for 1 h. After unbound antibodies were removed by washing three times with PBS, the nitrocellulose membranes were incubated for 1 h with horseradish peroxidase-conjugated protein A. The PLC-β isoenzyme bands were identified with the use of enhanced chemiluminescence reagents.

Measurement of IP₃ production. Extraction and HPLC separation of IP₃ were performed as previously described (43, 48). Freshly obtained gallbladder muscle squares were incubated with 60 µCi/ml myo-[2-³H]inositol at 37°C for 4 h. The tissue was collected and evenly divided into the experimental and control tubes. The experimental groups were exposed to CCK-8 (1 mM) for 10 s, and the control groups were exposed to Krebs solution only. The reaction was quenched by the addition of an ice-cold chloroform-methanol-HCl mixture (100:50:1, vol/vol). Phytic acid (25 µl at 100 mg/ml) was added to each tube to enhance HPLC signals. The aqueous cytosolic phase was separated by centrifugation, collected three times, and neutralized to pH 6.5–7.5. Samples were passed through a Partisil 10 SAX HPLC column (Whatman, Clinton, NJ), which was connected to a HPLC system (Bio-Rad, Richmond, CA). The phosphate-containing inositol phosphate metabolites were selectively eluted in a stepwise fashion with 1.5 M ammonium formate buffer as previously described (43, 48). The HPLC column eluate was pumped into the detector system, where it was mixed with FLO-SCINT IV scintillation fluid (Packard Instrument, Downers’ Grove, IL) and pumped through a 1-ml flow cell for radiochemical detection. Radioactivity was determined by continual flow liquid scintillation counting using a FLO-ONE Beta Radiochromatography Detector Series A-200 (Radiomatic Instruments Chemical, Tampa, FL). Five major radioactive peaks were isolated with the use of the elution pattern listed above. Among them, three could be identified as inositol 1-monophosphate (IP₁), inositol 1,4-bisphosphate (IP₂), and IP₃, according to the standard [³H]inositol phosphatemarker (Amer sham, Arlington Heights, IL). Values were expressed as disintegrations per minute (dpm) per milligram protein.

Extraction and quantitation of DAG. DAG was extracted and measured as previously described with some modifications (34, 48). The gallbladder muscle squares were equilibrated in 450 µl Krebs solution for 20 min at 37°C. Aliquots of

Immunoblot analysis of PLC-β isoenzymes in gallbladder muscle. Western blot analysis of PLC-β isoenzymes was performed as described previously (26, 28). Gallbladder muscle squares were homogenized in medium containing 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1% Triton X-100, 2 mM PMSF, 0.1 mM dithiothreitol (DTT), and 2 µg/ml leupeptin and centrifuged at 12,000 × g for 5 min. The supernatant was loaded and subjected to 8% SDS-PAGE (Mini-PROTEAN II cell; Bio-Rad, Hercules, CA). Prestained molecular weight markers were run in an adjacent lane to allow molecular weight to be determined. The separated proteins were electrical transferred at 4°C to a nitrocellulose membrane (Bio-Rad, Melville, NY) in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. The nitrocellulose membranes were blocked with 5% dry nonfat milk blocking 0.2% Tween-20 at room temperature for 1 h, followed by incubation with different anti-PLC-β isoenzyme-specific antibodies (1 µg/ml) for 1 h. After unbound antibodies were removed by washing three times with PBS, the nitrocellulose membranes were incubated for 1 h with horseradish peroxidase-conjugated protein A. The PLC-β isoenzyme bands were identified with the use of enhanced chemiluminescence reagents.

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The use of the Tri-Carb 1900 CA liquid scintillation analyzer were developed in a solvent consisting of chloroform-methanol (Gibbstown, NJ) activated by running in acetone. TLC plates spotted on a 20-cm Silica Gel 60 TLC plate (EM Separations, methanol in chloroform, and 20 µl of the lower phase were twice with 1 ml of 1% perchloric acid. The lower phase was discarded and the lower organic phase was washed with a Polytron and 60 strokes of a Dounce Grinder. Aliquots contained (in mM) 20 Tris (pH 7.5), 0.5 EDTA, 0.5 mM DTT, and 10 µl DAG kinase. The reaction was initiated by adding 10 µl of 5 mM [γ-32P]ATP (sp act 22.2 dpm/pmole) prepared in 100 mM imidazole and 1 mM DTPA, pH 6.6. It proceeded at 25°C for 30 min and was stopped by the addition of 20 µl of 1% perchloric acid. Lipid was extracted by adding 450 µl chloroform-methanol (1:2, vol/vol) and separated with the addition of 150 µl chloroform and 1% perchloric acid, followed by centrifugation at 20,000 g for 3 min. The upper phase was discarded and the lower organic phase was washed twice with 1 ml of 1% perchloric acid. The lower phase was then dried under nitrogen and redissolved in 60 µl of 5% methanol in chloroform, and 20 µl of the lower phase were spotted on a 20-cm Silica Gel 60 TLC plate (EM Separations, Gibbstown, NJ) activated by running in acetone. TLC plates were developed in a solvent consisting of chloroform-methanol-acetic acid (65:15:5, vol/vol/vol), air-dried, and subjected to radioautography. The radioactive spot corresponding to phosphatidic acid was scraped into a scintillation vial to which 10 ml of Ecolmue scintillation fluid (ICN Biomedicals, Irvine, CA) was added, and the radioactivity was quantitated with the use of the Tri-Carb 1900 CA liquid scintillation analyzer (Packard Instrument). The amount of DAG present in the sample was calculated from the amount of [32P]phosphatidic acid produced by the reaction of 10 µl of 5× 32P-labeled substrate solution containing 100 µM Ac-AMP (4—14), 100 µM ATP, 5 mM CaCl2, and 100 µM MgCl2 in 20 mM Tris with 0.5 mM EDTA and 0.5 mM EGTA (final volume 50 µl). The reaction was allowed to proceed at 30°C for 5 min. Then, aliquots of 25 µl were removed and spotted on to a P81 ion-exchange phosphocellulose paper (Whatman). The phosphocellulose paper was washed twice with 1% (vol/vol) phosphoric acid and twice with distilled water (5 min each). The remaining 32P activity on the phosphocellulose paper was quantitated with the use of the Tri-Carb 1900CA liquid scintillation analyzer. Data were expressed as picomoles per minute per milligram protein.

Protein determination. Protein content in each tissue sample was measured according to the method of Bradford, using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Mil- ville, NY). Values were means of triplicate measurements for each sample.

Drugs and chemicals. Type II collagenase and soybean trypsin inhibitor were purchased from Worthington Biochemicals (Freehold, NJ). Polyclone antibodies to Goα1, Goα3, Gβ1/2, Gβ3, and Gγ-subunits and PTx were obtained from Calbiochem (La Jolla, CA); polyclonal antibodies to PLC-β2 and PLC-β3 were from Santa Cruz Biochemicals (Santa Cruz, CA); and monoclonal antibody to PLC-β1 was obtained from Upstate Biotechnology (Lake Placid, NY). Recent studies have demonstrated the ability of these G protein and PLC-β isoenzyme antibodies to block activation or inhibition of specific effector enzymes (11, 29, 40). CCK-8 was purchased from Bachem (Torrance, CA). [35S]GTP-γ-S was purchased from DuPont-New England Nuclear (Boston, MA). Horseradish peroxidase-conjugated protein A, enhanced chemiluminescence reagents, and rainbow prestained molecular markers were obtained from Amersham. 1-(5-Isouquinolinylsulfonyl)-2-methyl-piperazine (H-7) was purchased from Sekagaku America (St. Petersburg, FL). 1,3-Dihydroxy-1-[(4-methyl-4H,6-H-phenyl)cyclopenta-1,2-(4,1)benzoazepin-4-yl][methyl-4-piperidinyl]2H-benzimidazole-2-1-maleate (CGS9343B) was a gift from Richard A. Lovell of Ciba-Geigy (Summit, NJ). 1-(6-[17p]-3-Methoxyestra-1,3,5(10)-trien-17-yl)methoxyhexyl-
CCK-8 was reduced by 72% from that in a normal Ca²⁺ contraction, since the 4 mM Sr²⁺ extracellular Ca²⁺ caused by the maximally effective dose of KCl (Fig. 1). KCl has been shown to utilize only contraction caused by the maximally effective dose of KCl in both muscle strips (21). The Ca²⁺-free medium, however, abolished the contraction caused by the maximally effective dose of KCl (Fig. 1). KCl has been shown to utilize only extracellular Ca²⁺ by depolarizing the plasma membrane and inducing Ca²⁺ influx (21). When muscle cells were preincubated in a Ca²⁺-free medium containing 2 mM EGTA for 60 min, their maximal shortening in response to 10 nM CCK-8 was 26 ± 3% and was not significantly different from that in normal Ca²⁺ medium. The Ca²⁺-free medium, however, abolished the activation of specific effector enzymes has been demonstrated in previous studies (11, 40). The maximal contraction induced by 10 nM CCK-8 in permeabilized gallbladder muscle cells was significantly inhibited by pretreating muscle cells with PTx, anti-G₁₃α, and anti-β₁γ-subunit-specific antibodies, but not by pretreating with anti-Gα₁₂-3, Gγ₅γ₇, or Gγ₁₂α-specific antibodies (Fig. 2). These data suggest that in gallbladder muscle the G proteins activated by CCK-8 are PTx-sensitive α- and β₁γ-subunits of G₁₃ protein. To further confirm this finding [³⁵S]GTP₇S binding induced by CCK-8 stimulation was performed using specific G protein antibodies to immunoprecipitate the activated specific G proteins. As shown in Fig. 3, CCK-8 (1 µM) caused a significant increase in [³⁵S]GTP₇S binding to G₁₃ but not to Gα₁₂-3 and Gγ₁₂α. This increased binding was completely blocked by pretreatment of muscle membranes with PTx.

Activation of PLC-β isoforms by CCK. To examine the roles of the phospholipases, we tested the effects of a PLC inhibitor, U-73122, and a phosphatidic acid phosphohydrolase (which produces DAG through the

RESULTS

Ca²⁺ source utilized by CCK. CCK-8 (0.1 pM–10 nM) contracted the intact and permeabilized gallbladder muscle cells in a dose-dependent manner (P < 0.01, by one factorial ANOVA). Contraction reached a plateau at about 26 ± 1% shortening at 10 nM CCK-8. There was no significant difference in contractility between intact and permeabilized muscle cells. When muscle cells were preincubated in a Ca²⁺-free medium containing 4 mM Sr²⁺ instead of Ca²⁺ for 60 min, their response to 10 nM CCK-8 was reduced by 72% from that in a normal Ca²⁺ medium. The Ca²⁺-free medium, however, abolished the contraction caused by KCl in both muscle strips (21) and single muscle cells.

Identification of CCK receptor-activated G protein. To examine the G protein subtype functionally coupled with the CCK receptor, we preincubated muscle cells with PTx at 0.5 and 1.6 µg/ml or with different G protein subunit antibodies (1:400) before exposure to CCK. The ability of these G protein antibodies to block activation of specific effector enzymes has been demonstrated in previous studies (11, 40). The maximal contraction induced by 10 nM CCK-8 in permeabilized gallbladder muscle cells was significantly inhibited by pretreating muscle cells with PTx, anti-G₁₃α, and anti-β₁γ-subunit-specific antibodies, but not by pretreating with anti-Gα₁₂-3, Gγ₅γ₇, or Gγ₁₂α-specific antibodies (Fig. 2). These data suggest that in gallbladder muscle the G proteins activated by CCK-8 are PTx-sensitive α- and β₁γ-subunits of G₁₃ protein. To further confirm this finding [³⁵S]GTP₇S binding induced by CCK-8 stimulation was performed using specific G protein antibodies to immunoprecipitate the activated specific G proteins. As shown in Fig. 3, CCK-8 (1 µM) caused a significant increase in [³⁵S]GTP₇S binding to G₁₃ but not to Gα₁₂-3 and Gγ₁₂α. This increased binding was completely blocked by pretreatment of muscle membranes with PTx.
PLD-mediated pathway) inhibitor, propranolol, on CCK-8-induced contraction. U-73122 at 10 mM inhibited the contraction caused by the maximally effective dose (10 nM) of CCK-8 by 78%. It also inhibited the contraction induced by a low dose of CCK-8 (0.1 pM) by 77%. On the other hand, 10 µM propranolol had no effect on the contractions caused by the high and low concentrations of CCK-8 (Fig. 4).

We then determined the presence of PLC-β isoenzymes that are functionally coupled with CCK receptor in gallbladder muscle. Immunoblot analysis (Western blot) and muscle cell contraction were performed using a panel of specific PLC-β isoenzyme antibodies. The ability of these antibodies to block activation of PLC-β enzymes has been demonstrated in recent studies (11, 29). Figure 5 shows the presence of PLC-β2 and PLC-β3 but not PLC-β1 in gallbladder muscle. In contrast, a full complement of PLC-β isoenzymes are present in the ileal circular smooth muscle, which is consistent with previously reported studies (28). The maximal contraction induced by 10 nM CCK-8 in permeabilized gallbladder muscle cells was significantly inhibited by pretreating muscle cells with anti-PLC-β3-specific antibody (10 µg/ml), but not anti-PLC-β1- or PLC-β2-specific antibodies (Fig. 6A). In contrast, in cat ileal circular muscle cells CCK-8-induced contraction was selectively blocked by anti-PLC-β1-specific antibody but was unaffected by anti-PLC-β2 or PLC-β3 antibodies (Fig. 6B).

The role of IP3 in mediating CCK-induced contraction. To examine the role of IP3 in CCK-induced contraction in gallbladder muscle, the effect of low molecular weight heparin, a specific IP3 receptor antagonist, on

CCK-8 was tested. Permeabilized cells were preincubated with heparin for 60 s before CCK-8 was added. The maximal contraction caused by 10 nM CCK-8 was reduced by heparin in a concentration-dependent manner (Fig. 7A). Maximal inhibition of 84% was reached at a heparin concentration of 10 µM. The effect of exogenous IP3 on gallbladder muscle contraction was also tested. Like heparin, IP3 does not diffuse across the plasma membrane. Therefore, it was used in permeabilized cells as well. IP3 contracted the permeabilized muscle cells in a dose-dependent manner (0.1 nM–1 µM), and peak contraction (21 ± 1%) was obtained at 1 mM IP3 (Fig. 7B).

To further support the above findings, production of IP3 and DAG was measured in gallbladder muscle after CCK-8 stimulation. The gallbladder muscle squares were exposed to 1 mM CCK-8 for 10 s. We have previously shown that the concentrations of CCK needed to contract the gallbladder muscle strips (similar to muscle squares) are 3–4 log M higher than that needed to contract the single cells (21). CCK-8 at 1 mM increased IP3 production by 94% from the basal level (Fig. 8). DAG production was measured at 15, 30, 60, and 90 s after 1 mM CCK-8 stimulation. Preliminary data showed that DAG started to increase at 15 s,
reached a peak at 30 s, slightly decreased at 60 s, and
remained elevated for up to 90 s (data not shown). DAG
production at 30 s after CCK-8 stimulation is shown in
Fig. 8. CCK-8 at 1 mM increased DAG production by
86% from the basal level.

PKC activation in CCK-induced contraction. When
Ca\(^{2+}\) is released from intracellular stores by IP3, it can
either activate calmodulin-MLCK or potentiate DAG to
activate PKC (12, 20, 31–32, 42). To examine the roles of
calmodulin and PKC in mediating gallbladder muscle
contraction, we tested the effects of the calmodulin
antagonist CGS9343B and the PKC inhibitor H-7 on
CCK-8-induced contraction and measured PKC activity
in the cytosolic and membranous fractions of gallblad-
der muscle. CGS9343B at 10 mM reduced the maximal
contraction caused by 10 nM CCK-8 (23.4 ± 1.4%) to
5.6 ± 2.0%. CGS9343B, however, had no effect on
contraction caused by a low dose (0.1 pM) of CCK-8. In
contrast, H-7 at 10 µM had no effect on contraction
caused by 10 nM CCK-8, but it abolished the contrac-
tion caused by 0.1 pM CCK-8 (Fig. 9).

These findings were further confirmed by the direct
measurements of PKC activity. The gallbladder muscle
squares were exposed to low (10 nM) and high (1 µM)
concentrations of CCK-8 for 2 min. As we mentioned
above, the concentrations used in muscle strips or
squares are different from the concentration used in
single cells. The low dose of CCK-8 (10 nM) decreased
basal PKC activity in the cytosolic fraction from 29.2 ±
2.3 to 17.6 ± 5.2 pmol·min\(^{-1}\)·mg protein\(^{-1}\) and
increased basal PKC activity in the membranous fraction
from 13.0 ± 1.3 to 22.8 ± 2.4 pmol·min\(^{-1}\)·mg protein\(^{-1}\),
leading to a significant increase in the ratio of membra-
nous to cytosolic fraction, i.e., PKC translocation (Fig.
10). The high dose of CCK-8 (1 µM), however, caused no
changes of PKC activity in either fraction.

DISCUSSION

The results of the present study support our previous
finding (21) that CCK causes gallbladder muscle con-
traction in muscle strips by utilizing intracellular Ca\(^{2+}\),
since the contraction was blocked by Sr\(^{2+}\) replacement
for Ca\(^{2+}\) in the medium and was not affected by a
Ca\(^{2+}\)-free medium. It appears that these two media are
able to distinguish the Ca\(^{2+}\) sources utilized by ago-
nists. In both preparations of gallbladder muscle strips
and single cells, we were able to show that a Ca\(^{2+}\)-free
medium abolished the contraction caused by KCl. Potas-
sium contracts the smooth muscle cells by depolarizing
plasma membranes and opening Ca\(^{2+}\) channels, result-
ing in increased Ca\(^{2+}\) influx. Sr\(^{2+}\) displaces Ca\(^{2+}\) in the
endoplasmic reticulum but cannot be released from the
intracellular stores (41, 47). The finding that Sr\(^{2+}\) does
not affect KCl-induced contraction suggests that it can
only replace the role of extracellular Ca\(^{2+}\). Indeed, the
conductance and permeability of the L-type Ca\(^{2+}\) chan-
nel to Sr\(^{2+}\) and Ca\(^{2+}\) are closely similar (38, 45).

It has been shown in most studies that CCK activates
G\(_{q/11}\) proteins. In the circular muscle of the guinea pig ileum, CCK-induced contraction is mediated by activa-
tion of a PTx-insensitive G_{i1alpha} subunit that results in stimulation of PLC-1 (26). However, it has also been reported that CCK can activate G_i and G_{i3} proteins (37). In the present study, our data showed that the contraction induced by CCK in gallbladder muscle was blocked by PTx and by anti-\(\alpha_{i3}\), anti-\(\beta_1\)-subunit-, and anti-PLC-\(\beta_3\)-specific antibodies, suggesting that CCK receptors in gallbladder muscle are selectively coupled to PLC-\(\beta_3\) via both \(\alpha_1\) and \(\beta_1\)-subunits of G_{i3} protein. It thus appears that different pathways couple to CCK receptors in the gallbladder and ileal circular muscle. These differences therefore appear to be tissue specific rather than peptide specific and are further supported by the immunoblot analysis, which shows only PLC-\(\beta_3\)-subunits, anti-\(\alpha_1\)-subunit-, and anti-\(\beta_1\)-subunit-specific antibodies, suggesting that CCK receptors in gallbladder muscle are selectively coupled to PLC-\(\beta_3\) via both \(\alpha_1\) and \(\beta_1\)-subunits of G_{i3} protein. It thus appears that different pathways couple to CCK receptors in the gallbladder and ileal circular muscle. These differences therefore appear to be tissue specific rather than peptide specific and are further supported by the immunoblot analysis, which shows only PLC-\(\beta_3\)-subunits, anti-\(\alpha_1\)-subunit-, and anti-\(\beta_1\)-subunit-specific antibodies, suggesting that CCK receptors in gallbladder muscle are selectively coupled to PLC-\(\beta_3\) via both \(\alpha_1\) and \(\beta_1\)-subunits of G_{i3} protein.

Our findings also suggest that PLC-\(\beta_3\) mediates CCK-induced contraction by hydrolyzing PIP_2 into IP_3 and DAG. They further showed that 1) IP_3 contracts the permeabilized gallbladder muscle in a dose-dependent manner, suggesting the existence of IP_3 receptors in the endoplasmic reticulum. Similar findings have been observed in guinea pig ileum (26). 2) The actions of CCK were blocked in a dose-dependent manner by low molecular weight heparin (3, 4, 8, 10, 18). It is known that heparin blocks IP_3 competitively at receptors that mediate Ca^{2+} release from Ca^{2+}-stores. 3) CCK stimulation generates IP_3 and DAG. Direct measurements of IP_3 and DAG showed a nearly twofold increase in these second messengers at 10 s. IP_3 is the exclusive product of PIP_2 hydrolysis by PLC (13, 27).

DAG, however, can also be formed by the hydrolysis of phosphatidylcholine by phospholipase D (PLD), which forms phosphatidic acid. Phosphatidic acid is further hydrolyzed to DAG by phosphohydrolase (5, 14). Therefore, the effects of the PLC and phosphohydrolase antagonists U-73122 and propranolol, respectively, were tested to determine whether the CCK-induced contraction was also mediated by PLD. U-73122 competes with Ca^{2+} for binding to the site on PLC that must be occupied by Ca^{2+} for expression of PLC activity, and therefore inhibits agonist-induced IP_3 production (9, 39). Propranolol inhibits phosphatidic acid phosphohydrolase, preventing the formation of DAG through the PLD-mediated pathway (7, 35). The finding that U-73122, but not propranolol, inhibited the contraction induced by CCK supports the conclusion that PLC, not PLD, plays a role in the CCK action on gallbladder muscle.

Ca^{2+} released from the storage sites may activate calmodulin (12, 20, 42) by affecting PKC directly or by its synergistic action with DAG (31, 32). To characterize these final steps in the signal-transduction pathways leading to muscle contraction, we tested the effects of a calmodulin antagonist, CGS9343B, and a PKC inhibitor, H-7, on CCK-induced contraction and measured the PKC activity after CCK stimulation. The data show that low concentrations of CCK (i.e., 0.1 pM for single muscle cells and 10 nM for muscle strips and squares) caused a small contraction, which was blocked by H-7 but not by CGS9343B, and caused a significant PKC translocation from the cytosol to the membrane, an indication of PKC activation (1, 16). High concentrations of CCK (i.e., 10 nM for single cells and 1 mM for muscle strips and squares) caused a contraction that was not affected by H-7 but was blocked by CGS9343B. These CCK concentrations caused no PKC translocation. One possibility is that low doses of CCK utilize an alternative pathway (PLD) to produce DAG, resulting in the activation of PKC, whereas a high dose of CCK activates PLC, forming IP_3, releasing Ca^{2+}, and activating calmodulin. However, it is unlikely that PLD is involved in the contraction caused by low doses of CCK, since the contraction was not affected by propranolol but was inhibited by U-73122. Therefore, the factor that determines this switch may be the amount of Ca^{2+} released from intracellular stores.

Although the intracellular Ca^{2+} concentrations in these experiments were unknown, it has been shown in other tissues that CCK and IP_3 release Ca^{2+} from intracellular stores in a concentration-dependent manner, which correlates with the dose-dependent contraction in response to the same agonist (22, 26). With a low dose of CCK, low levels of IP_3 and DAG are presumably formed by the activation of PLC. When a small amount of Ca^{2+} is released by low levels of IP_3, it could potentiate DAG to activate PKC but may not be sufficient to activate calmodulin by itself. It is known that PKC has a greater affinity for Ca^{2+} than calmodulin (5, 48). This assumption is also supported by our previous findings that the potentiation with low doses of exogenous IP_3 and DAG was blocked by H-7 but was unaffected by CGS9343B (48). With high doses of CCK, larger amounts of Ca^{2+} are presumably released, which may be able to activate calmodulin, and the calmodulin activation appears to inhibit the activation of PKC. This is supported by the finding that exogenously activated calmodulin inhibited the contraction caused by the PKC activator DAG (48). The inhibition of PKC activity by calmodulin has been shown in several other tissues and at different levels, i.e., at the kinase itself or at the substrate level (19, 30, 49). Our results suggest that activated calmodulin inhibits the activation of PKC in gallbladder muscle.

In summary, in gallbladder muscle cells CCK activates PTx-sensitive G_{i3alpha} protein coupled with PLC-\(\beta_3\) to produce IP_3 and cause intracellular Ca^{2+} release. Depending on the concentration, CCK could activate either PKC or calmodulin, leading to gallbladder muscle cell contraction; low doses of CCK activate the PKC-dependent pathway, whereas high doses of CCK activate the calmodulin-dependent pathway.

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