Action of Cholecystokinin and Cholinergic Agents on Calcium Transport in Isolated Pancreatic Acinar Cells

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ABSTRACT COOH-terminal octapeptide of cholecystokinin (CCK-octapeptide) and the cholinergic agent carbamylcholine each produced a fourfold stimulation of calcium outflux in guinea pig isolated pancreatic acinar cells. Neither agent altered calcium influx. Stimulation of calcium outflux was rapid and specific, was abolished by reducing the incubation temperature to 4°C, and was a saturable function of the secretagogue concentration. The concentrations of CCK-octapeptide and carbamylcholine that produced half-maximal stimulation of calcium outflux were 3.1×10^{-10} M and 4.9×10^{-5} M, respectively. The cholinergic antagonist atropine competitively inhibited carbamylcholine stimulation of calcium outflux but did not alter stimulation produced by CCK-octapeptide. Stimulation of calcium outflux by maximal concentrations of carbamylcholine plus CCK-octapeptide was the same as that produced by a maximal concentration of either agent alone. Calcium outflux became refractory to stimulation by secretagogues, and incubation with either CCK-octapeptide or carbamylcholine produced a refractoriness to both agents. The relative potencies with which CCK and its related fragments stimulated calcium outflux were CCK-octapeptide > heptapeptide > CCK > hexapeptide = gastrin. Secretin, glucagon, and vasoactive intestinal peptide, at concentrations as high as 10^{-5} M, failed to alter calcium outflux and did not affect stimulation by CCK-octapeptide or by carbamylcholine.

INTRODUCTION

The peptide hormone cholecystokinin (CCK)[1], as well as cholinergic agents such as acetylcholine and carbamylcholine, are known to stimulate enzyme secretion from the pancreas in vivo, *in situ,* and in vitro (1–3). Although the sequence of morphologic changes attending pancreatic enzyme secretion has been characterized (4), the coincident sequence of biochemical events is poorly understood and the mechanism by which CCK and cholinergic agents initiate pancreatic secretion has not been characterized. Some investigators (5-10) have suggested that stimulation of cellular accumulation of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) is involved in stimulation of pancreatic enzyme secretion, while others (11-22) have focused on changes in membrane transport of cations, in general, and of calcium in particular.

A major requirement for elucidating the biochemical mechanism of action of pancreatic secretagogues is a homogeneous preparation of isolated pancreatic cells that maintain their viability and hormone responsiveness in vitro for a sufficient period of time. Amsterdam and Jamieson (23) have reported a technique for preparing viable, isolated acinar cells from guinea pig pancreas that respond to secretagogues in vitro for several hours. In the present studies we have used isolated acinar cells from guinea pig pancreas to explore the kinetics, stoichiometry, and chemical specificity with which CCK and carbamylcholine alter membrane transport of calcium, in an effort to better understand the mechanism of action of these secretagogues and to establish a basis for directly evaluating hormone receptor interaction as well as for exploring the role of calcium in pancreatic enzyme secretion.

METHODS

Male Hartley strain albino guinea pigs (350–400 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md. ^{45}Ca (12.5 mCi/mg) was purchased from New England Nuclear, Boston, Mass.; synthetic human gastrin I and gastrin II from Imperial Chemical Industries Limited, London, England; synthetic porcine secretin from Schwarz/Mann

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[1] *Abbreviations used in this paper:* BOC, butyloxy carbonyl; CCK, cholecystokinin; VIP, vasoactive intestinal peptide.

Div., Becton, Dickinson & Co., Orangeburg, N. Y.; pentagastrin (Peptavlon) from Ayerst Laboratories, New York; and porcine glucagon from Eli Lilly and Company, Indianapolis, Ind. Natural porcine secretin, natural porcine vasoactive intestinal peptide (VIP), and natural porcine CCK were gifts from Prof. Viktor Mutt, GIH Research Unit, Karolinska Institutet, Stockholm, Sweden. Prostaglandin E_1 was a gift from Dr. John Pike, The Upjohn Company, Kalamazoo, Mich. Crude collagenase (*Clostridium histolyticum*, EC 3.4.4.19) and crude bovine-testis hyaluronidase (EC 3.2.1.35) were purchased from Sigma Chemical Co., St. Louis, Mo., and chromatographically purified soybean trypsin inhibitor was purchased from Worthington Biochemical Corp., Freehold, N. J. Bovine plasma albumin, fraction V, was purchased from Miles Laboratories, Inc., Elkhart, Ind. All other reagents were of the highest grade commercially obtainable.

Tissue preparation. After an overnight fast, animals were killed by cervical dislocation. The pancreas was dissected free of fat and mesentery, and isolated pancreatic exocrine cells were prepared by the procedure of Amsterdam and Jamieson (23). Cells were isolated by incubating one pancreas (0.8 g of tissue) in a Krebs-Ringer bicarbonate buffer (pH 7.4), equilibrated with 95% O_2 and 5% CO_2, and containing L-amino acid supplement (24), 0.1 mg/ml soybean trypsin inhibitor, and 14 mM glucose. Crude collagenase and hyaluronidase, $MgCl_2$, $CaCl_2$, and disodium EDTA were added as previously described (23). The tissue was incubated at 37°C in a Dubnoff shaking metabolic incubator (130 oscillations/min). The entire isolation procedure required approximately 90 min from the time the animals were sacrificed. At the end of the incubation cells were liberated by passage through a Pasteur pipet five times. The cells were layered over Krebs-Ringer bicarbonate (pH 7.4) containing 4% (wt/vol) albumin, 1.0 mM $CaCl_2$, and centrifuged at 50 *g* for 5 min at 4°C. This procedure was repeated twice and then performed once with buffer containing 8% albumin and 0.5 mM calcium. By light and electron microscopy more than 96% of the cells were zymogen-containing exocrine cells. Viability of these cells was demonstrated by their ability to exclude trypan blue, to incorporate L-[^{3}H]leucine into cellular protein, and to maintain their cellular concentrations of sodium and potassium for up to 4 h.

^{45}Ca transport. Uptake of ^{45}Ca by isolated guinea pig pancreatic acinar cells was determined with the technique used previously to measure transport of other substrates and ions in other isolated cell preparations (25–30). Pancreatic acinar cells (5–20 × 10^6 cells/ml) were incubated at 37°C in the standard buffer, Krebs-Ringer bicarbonate (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca. At appropriate times cellular ^{45}Ca was measured by taking duplicate 100-μl samples and the cells were washed three times with 300 μl of iced (4°C) buffer containing no ^{45}Ca. Washing was accomplished by alternate centrifugation for 15 s at 10,000 *g* with a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and resuspension. 100 μl of 10% (vol/vol) perchloric acid was added to the washed cells. The mixture was agitated, and centrifuged at 10,000 *g* for 45 s and the supernate was dispersed into 20 ml of liquid scintillation solution (15 parts toluene, J. T. Baker Chemical Co., Phillipsburg, N. J.; 5 parts Triton X-100, New England Nuclear; 1 part Liquifluor, New England Nuclear). Liquid scintillation counting was performed with a Packard model 3320 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

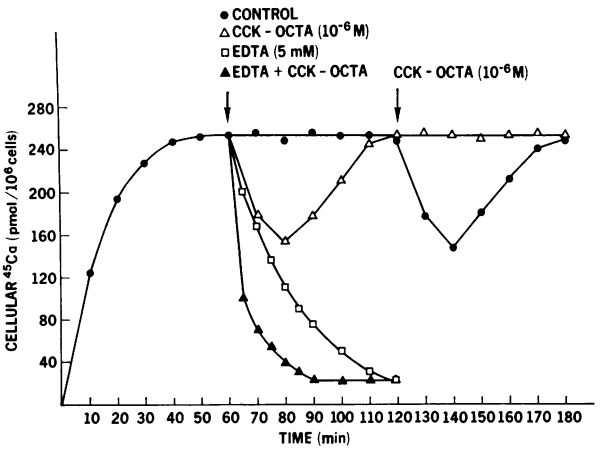

FIGURE 1 Effect of COOH-terminal octapeptide of cholecystokinin (CCK-octa) on cellular ^{45}Ca in guinea pig pancreatic acinar cells. Isolated cells were incubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca at 37°C. After 60 min EDTA and/or CCK-octa was added and cellular ^{45}Ca determined at intervals during a subsequent 60-min incubation. At the end of this second incubation period, CCK-octa was added to the control incubation and to the cells incubated with CCK-octa, and cellular ^{45}Ca was determined at intervals during a subsequent 60-min incubation. Each point is the mean of quadruplicate determinations and this experiment is representative of nine others.

Amylase release. To determine amylase release, isolated pancreatic cells (0.5–1 × 10^6 cells/ml) were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.5 mM $CaCl_2$ and 1% (wt/vol) albumin at 37°C. At zero and 20 min 100-μl samples of supernate were taken and assayed for amylase activity by the technique of Bernfeld (31). At some time during the incubation, 100 μl of the cell suspension were homogenized with 2.0 ml of 10 mM Tris Cl (pH 7.6) and amylase activity was determined on a sample of the lysate. Amylase release was expressed as the percent of the total amylase activity, as measured in the lysate, which appears in the incubation medium.

RESULTS

Uptake of ^{45}Ca by isolated guinea pig pancreatic acinar cells was moderately rapid, and cellular ^{45}Ca reached a steady state after 45 min of incubation (Fig. 1). After addition of synthetic COOH-terminal octapeptide of CCK (CCK-octapeptide, 10^{-6} M) to cells preloaded with the tracer for 60 min, cellular ^{45}Ca decreased rapidly, reached a minimum after 20 min and then steadily increased to control values during the subsequent 40 min. A similar response was produced by adding the peptide to cells preloaded with the tracer for 120 min. In cells incubated with ^{45}Ca for 60 min and then with 10^{-6} M CCK-octapeptide for 60 min, a second addition of fresh CCK-octapeptide (10^{-6} M) did not alter cellular ^{45}Ca. Adding 5 mM EDTA, which chelates extracellular ^{45}Ca and abolishes ^{45}Ca influx (i.e. the rate of

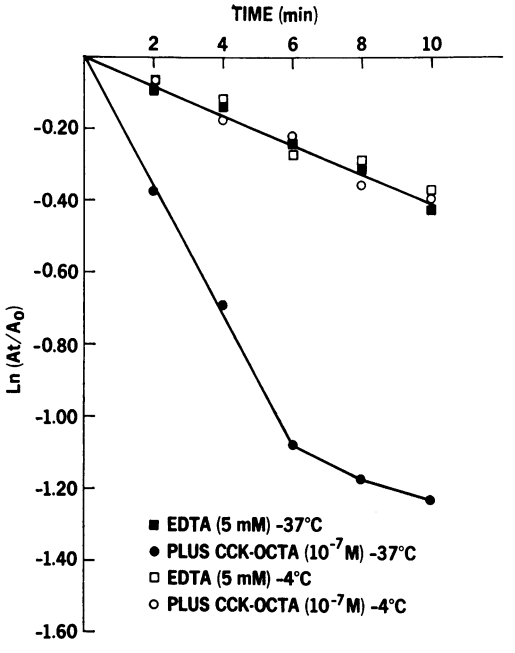

FIGURE 2 Effect of COOH-terminal octapeptide of cholecystokinin (CCK-octa) on the loss of ^{45}Ca from guinea pig pancreatic acinar cells. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.7 mM ^{45}Ca at 37°C for 50 min. EDTA with or without CCK-octa was added and cellular ^{45}Ca determined at intervals during a 10-min incubation at 37°C or 4°C. The natural logarithm of the fraction of cellular ^{45}Ca originally present is plotted as a function of time. Each point was determined in triplicate and results shown are the means from four separate experiments.

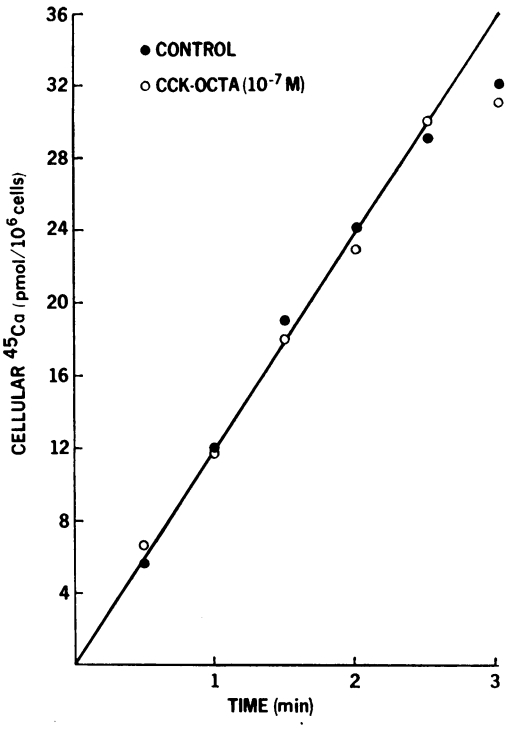

FIGURE 3 Uptake of ^{45}Ca by guinea pig pancreatic acinar cells. Isolated cells were preincubated for 60 min with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM calcium at 37°C. ^{45}Ca with or without CCK-octapeptide was added and cellular ^{45}Ca was determined at the indicated times during the incubation at 37°C. Each point was determined in duplicate and results shown are the means from four separate experiments.

unidirectional movement of ^{45}Ca from the incubation solution into the cells), produced a significant decrease in cellular ^{45}Ca and this decrease reflects ^{45}Ca outflux (i.e., the rate of unidirectional movement of ^{45}Ca from the cells into the incubation solution). Adding CCK-octapeptide (10^{-6} M) plus 5 mM EDTA accelerated the loss of ^{45}Ca from isolated pancreatic acinar cells, indicating that the octapeptide reduces cellular ^{45}Ca by stimulating calcium outflux.

Fig. 2 illustrates the loss of ^{45}Ca from pancreatic acinar cells during the initial few minutes after addition of EDTA (5 mM). A graph of the natural logarithm of the fraction of ^{45}Ca originally present in the cells was a linear function of time for 10 min and the slope of this line is the fractional rate of calcium outflux. With CCK-octapeptide (10^{-7} M) plus EDTA, a semilog plot of cellular calcium versus time was linear for 6 min, after which the slope decreased. During the initial 6-min incubation with EDTA, adding the octapeptide produced a fourfold stimulation of fractional calcium outflux. Reducing the incubation temperature from 37°C to 4°C did not alter basal calcium outflux but abolished the effect of CCK-octapeptide. In all subsequent experiments calcium outflux was calculated from the loss of cellular ^{45}Ca during a 5-min incubation with 5 mM EDTA at 37°C.[2]

Fig. 3 illustrates the uptake of ^{45}Ca by isolated pancreatic acinar cells during the initial few minutes of incubation. Cellular ^{45}Ca was a linear function of time for at least 2 min with or without CCK-octapeptide (10^{-7} M). These results indicate that cellular uptake

[2] In previous experiments using other types of cells (25, 26, 29), we have found it technically more convenient to preload cells with the tracer, wash them repeatedly with large volumes of iced (4°C) incubation solution containing no radioactivity, and after resuspending the cells in fresh medium, determine tracer outflux from the appearance of radioactivity in the extracellular solution. In the present study we found that ^{45}Ca outflux in isolated pancreatic cells could be determined by this technique; however, the precision and sensitivity were poor. That reducing the incubation temperature from 37°C to 4°C failed to reduce calcium outflux resulted in a significant loss of cellular radioactivity during the 15–20-min washing procedure used to remove extracellular radioactivity and in a relatively high amount of ^{45}Ca in the extracellular solution at the beginning of the outflux period.

of ^{45}Ca during a 2-min incubation can be used to determine calcium influx and that the octapeptide does not alter calcium influx in this system.

Stimulation of calcium outflux was a saturable function of the concentration of CCK-octapeptide (Fig. 4). Natural porcine secretin at concentrations as high as 10^{-5} M failed to stimulate calcium outflux or alter the stimulation produced by CCK-octapeptide. Similar results were obtained with synthetic porcine secretin, natural porcine VIP, or porcine glucagon at concentrations up to 10^{-5} M (data not shown). A double-reciprocal plot of stimulation of calcium outflux versus CCK-octapeptide concentration resulted in a straight line with an intercept on the *y*-axis significantly different from zero. The concentration of CCK-octapeptide that produced half-maximal stimulation of calcium outflux was 3.1 ($\pm$0.8) $\times 10^{-10}$ M (mean of five experiments $\pm$1 SD).

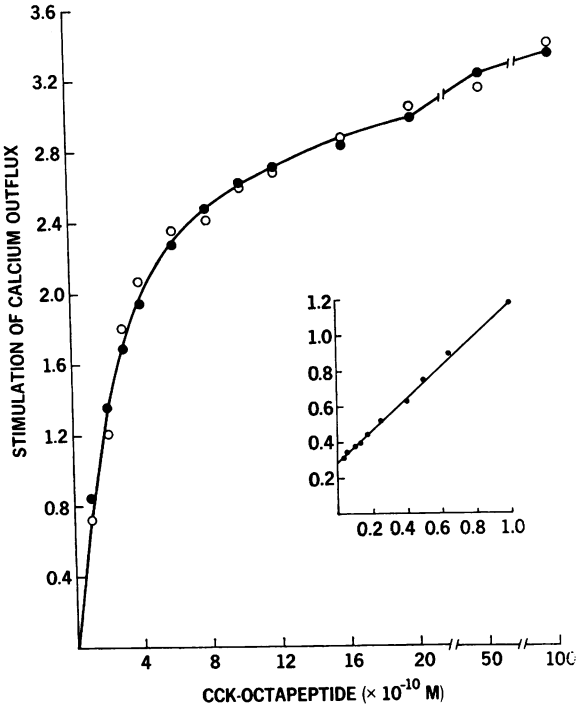

FIGURE 4 Stimulation of calcium outflux from guinea pig pancreatic acinar cells as a function of CCK-octapeptide concentration. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca for 60 min at 37°C. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 5-min incubation (at 37°C) after addition of 5 mM EDTA. Stimulation of calcium outflux was calculated as (hormone-control)/control. Closed circles represent results with CCK-octapeptide alone, while open circles represent results with CCK-octapeptide plus 10^{-5} M natural secretin. Each point was determined in triplicate and results given are the means of five separate experiments. The insert is a double-reciprocal plot of the values given in the main figure.

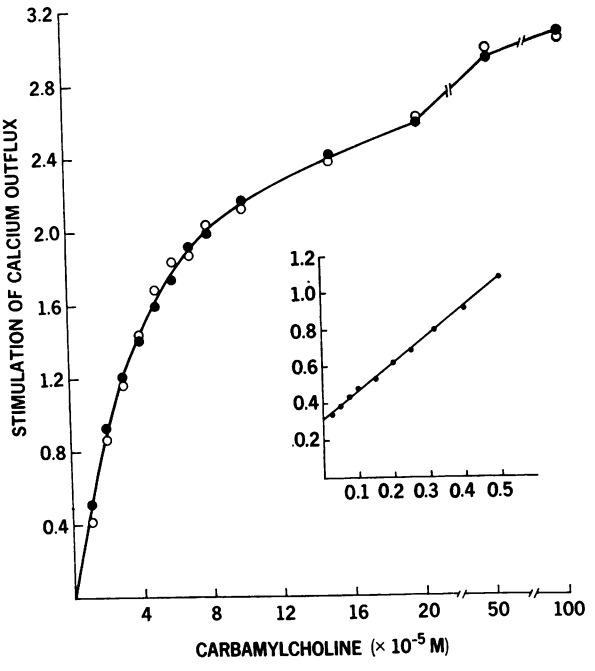

FIGURE 5 Stimulation of calcium outflux from guinea pig pancreatic acinar cells as a function of the concentration of carbamylcholine. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca for 60 min at 37°C. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 5-min incubation (at 37°C) after addition of 5 mM EDTA. Stimulation of calcium outflux was calculated as (agonist-control)/control. Closed circles represent results with carbamylcholine alone, while open circles represent results with carbamylcholine plus 10^{-5} M natural secretin. Each point was determined in triplicate and results given are the means of four separate experiments. The insert is a double-reciprocal plot of the values given in the main figure.

The cholinergic agent carbamylcholine also stimulated calcium outflux from isolated pancreatic acinar cells, and stimulation by carbamylcholine was not altered by concentrations of secretin as high as 10^{-5} M (Fig. 5). Stimulation of calcium outflux was a saturable function of the concentration of carbamylcholine, and a double-reciprocal plot of stimulation of calcium outflux versus carbamylcholine concentration gave a straight line with an intercept on the *y*-axis significantly different from zero. The concentration of carbamylcholine that produced half-maximal stimulation of calcium outflux was 4.9 ($\pm$1.6) $\times 10^{-5}$ M (mean of four experiments $\pm$1 SD). In addition to secretin, VIP and glucagon at concentrations from 10^{-9} M to 10^{-5} M failed to alter control calcium outflux or outflux stimulated by carbamylcholine (data not shown).

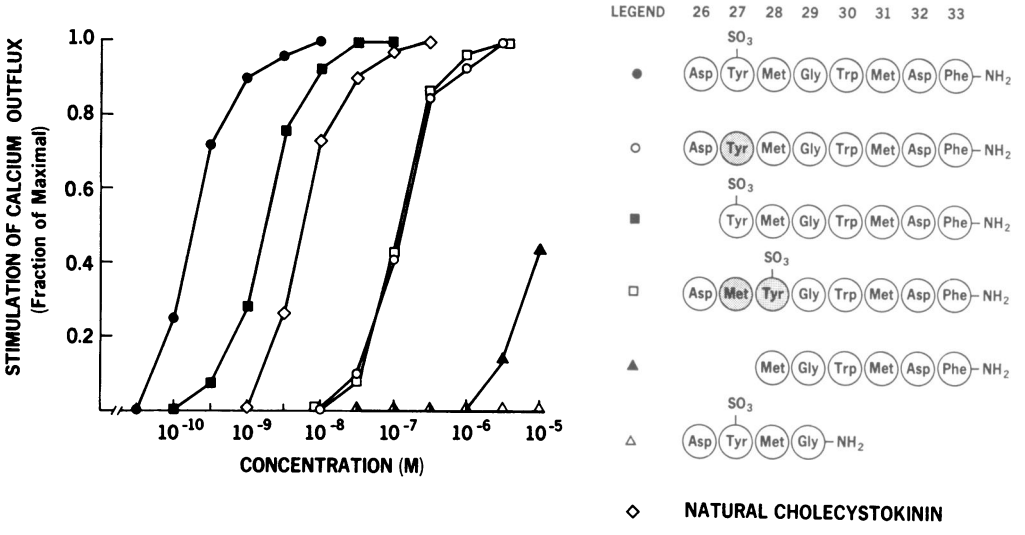

FIGURE 6 Stimulation of calcium outflux from guinea pig pancreatic acinar cells by natural CCK, fragments, and analogs. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca for 60 min at 37°C. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 5-min incubation (at 37°C) after addition of 5 mM EDTA. Stimulation of calcium outflux is given as the fraction of stimulation produced by 10^{-7} M CCK-octapeptide (i.e., fraction of maximal stimulation). Each point was determined in duplicate and results shown are means of at least four separate experiments.

The results in Figs. 6 and 7 illustrate the chemical specificity required for stimulation of calcium outflux in isolated pancreatic acinar cells by natural porcine CCK as well as by various fragments and analogs. Of

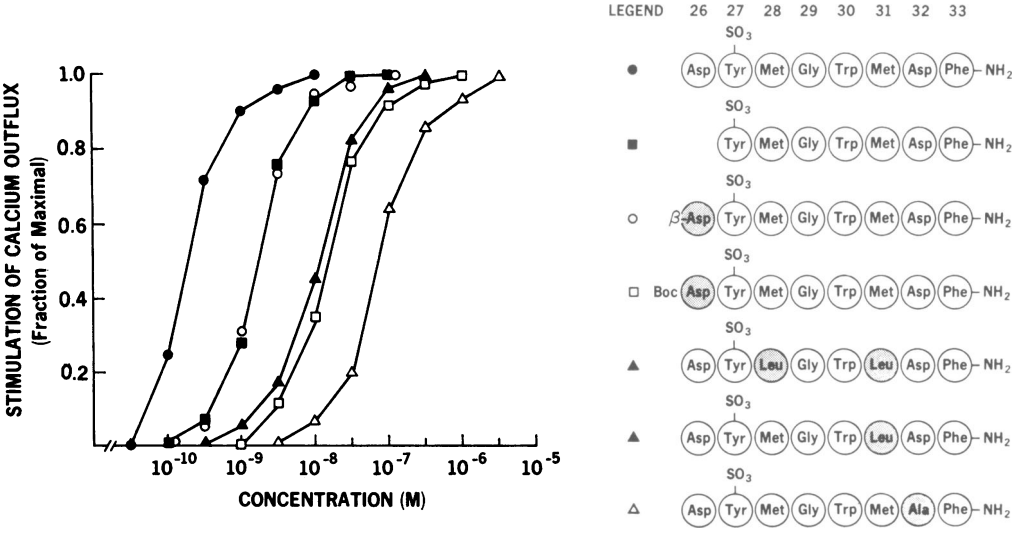

FIGURE 7 Stimulation of calcium outflux from guinea pig pancreatic acinar cells by fragments and analogs of natural CCK. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca for 60 min at 37°C. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 5-min incubation (at 37°C) after addition of 5 mM EDTA. Stimulation of calcium outflux is given as the fraction of stimulation produced by 10^{-7} M CCK-octapeptide (i.e. fraction of maximal stimulation). Each point was determined in duplicate and results shown are means of at least four separate experiments.

the peptides tested, CCK-octapeptide was the most potent (Fig. 6). Removing the aspartic residue from CCK-octapeptide reduced its potency by approximately 90%. The COOH-terminal octapeptide and heptapeptide were each more potent than the complete CCK molecule. Removing both the aspartic and the tyrosyl residues from CCK-octapeptide produced a 50,000-fold reduction in potency, and the stimulation produced by the COOH-terminal hexapeptide was identical to that produced by gastrin I, gastrin II, or pentagastrin. The tetrapeptide fragment 26-29-amide, at concentrations up to 10^{-5} M, failed to stimulate calcium outflux. Desulfation of the tyrosine moiety of CCK-octapeptide produced a 1,000-fold reduction in its potency and an identical effect was obtained by exchanging the sulfated-tyrosine residue with the adjacent methionine. Replacing the NH_2-terminal aspartic residue in CCK-octapeptide by β-aspartic results in a compound whose potency is the same as CCK-heptapeptide (i.e. 10% as potent as the octapeptide), while replacing the same residue with butyloxy carbonyl (BOC)-aspartic results in a compound whose potency is only 1% of that of CCK-octapeptide (Fig. 7). Replacing both methionine residues (positions 28 and 31) or only the methionine in position 31 with leucine resulted in compounds having 1% the potency of CCK-octapeptide. Finally, replacing the aspartic residue in position 32 by alanine resulted in a 500-fold reduction in the potency of CCK-octapeptide.

The stimulation of calcium outflux produced by maximal concentrations of CCK-octapeptide plus carbamylcholine was the same as that produced by a maximal concentration of either agent alone (Table I), i.e., stimulation of calcium outflux by CCK-octapeptide overlapped completely with that produced by carbamylcholine. The cholinergic antagonist atropine (10^{-4} M) did not alter basal calcium outflux in isolated pancreatic acinar cells or the stimulation of calcium outflux produced by CCK-octapeptide, but abolished the effect of carbamylcholine (Table I). The inhibition of carbamylcholine-stimulated calcium outflux by atropine increased with increasing concentrations of atropine and decreased with increasing concentrations of carbamylcholine, indicating that atropine is a competitive inhibitor of carbamylcholine (Fig. 8). From the results in Figs. 5 and 8, we calculated that the K_I[3] for atropine is 5.3 (±2.1) × 10^{-9} M (mean of four experiments ±1 SD).

Isolated pancreatic acinar cells, preincubated with CCK-octapeptide for 60 min, failed to show stimulation

TABLE I

Effect of CCK-Octapeptide and Carbamylcholine on Calcium Outflux from Isolated Pancreatic Acinar Cells

Additions	Calcium outflux
	%/min
None	4.3±0.4
Atropine (10^{-4} M)	4.2±0.3
CCK-octapeptide (10^{-7} M)	18.1±0.9*
plus atropine (10^{-4} M)	18.6±0.8*
Carbamylcholine (10^{-3} M)	17.2±1.1*
plus atropine (10^{-4} M)	4.6±0.3
CCK-octapeptide (10^{-7} M) plus carbamylcholine (10^{-3} M)	17.9±0.9*

Isolated pancreatic acinar cells were preloaded with 0.5 mM ^{45}Ca at 37°C with the standard incubation solution. Calcium outflux was determined from the loss of cellular radioactivity during a 5-min incubation with EDTA (final concentration 5 mM) plus the agents indicated. Results given are the means (±1 SD) of five separate experiments with each condition tested in each experiment.

* Significantly greater ($P < 0.01$) than calcium outflux with no additions by Student's t test.

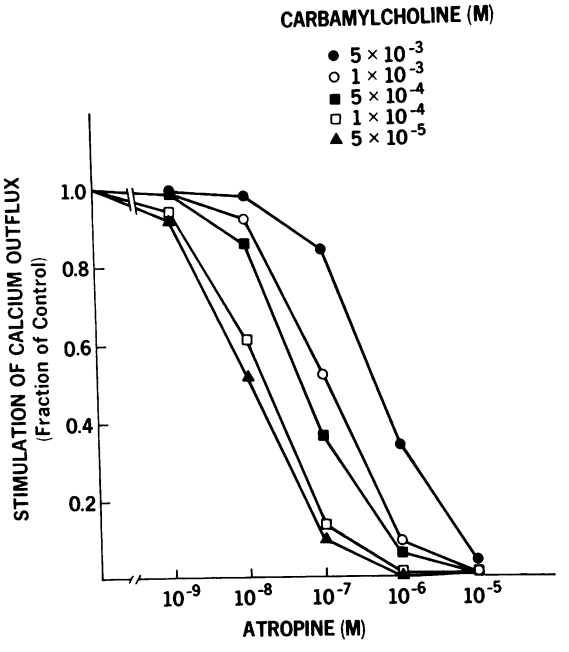

FIGURE 8 Inhibition of carbamylcholine-stimulated calcium outflux from guinea pig pancreatic acinar cells by atropine. Isolated cells were preincubated with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM ^{45}Ca for 60 min at 37°C. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 5-min incubation (at 37°C) after addition of 5 mM EDTA. Stimulation of calcium outflux was calculated as the fraction of stimulation obtained without atropine. Each point was determined in duplicate and results shown are means of four experiments.

[3] K_I is the concentration at which the antagonist (atropine) occupies 50% of the sites with which the secretagogue (carbamylcholine) can interact to stimulate calcium outflux (32).

TABLE II

Refractoriness of Calcium Outflux in Isolated Pancreatic Acinar Cells

Preincubation	Added during incubation	Calcium outflux
		%/min
None	None	4.8±0.3
None	Carb.	16.3±1.1*
None	CCK-octa	16.8±0.9*
Carb.	None	5.1±0.4
CCK-octa	None	4.6±0.5
Carb.	Carb.	4.7±0.4
Carb.	CCK-octa	5.0±0.3
CCK-octa	Carb	5.2±0.5
CCK-octa	CCK-octa	4.9±0.6

Isolated pancreatic acinar cells were preincubated at 37°C for 60 min in the standard incubation solution containing 0.5 mM ^{45}Ca plus the indicated agents. Calcium outflux was determined from the loss of cellular radioactivity during a 5-min incubation with 5 mM EDTA plus addition of the agents indicated. The concentrations of carbamylcholine (Carb.) and CCK-octapeptide (CCK-octa) were 10^{-3} M and 10^{-7} M, respectively. Results given are the means of four separate experiments ±1 SD.

* Significantly greater ($P < 0.01$) than calcium outflux with no additions by Student's *t*-test.

of calcium outflux with a second addition of CCK-octapeptide or with carbamylcholine (Table II). Similarly, preincubating isolated pancreatic cells with carbamylcholine abolished the stimulation of calcium outflux produced by carbamylcholine or by CCK-octapeptide.

In cells preincubated for 60 min, CCK-octapeptide and carbamylcholine each stimulated amylase release (three- to fourfold) (Table III). In contrast, cells preincubated for 10 min released amylase approximately five times more rapidly than cells preincubated for 60 min and showed no stimulation of amylase release by CCK-octapeptide or carbamylcholine. Calcium outflux, as well as its stimulation by CCK-octapeptide or carbamylcholine, was the same after 10 min of preincubation as after 60 min of preincubation.

DISCUSSION

The present study developed from our interest in preparing a homogeneous suspension of isolated pancreatic cells that could be maintained in vitro for several hours and which would maintain their hormone responsiveness. Isolated pancreatic cells prepared by the technique of Amsterdam and Jamieson (23) were found to meet these requirements and to consist of at least 96% acinar cells.

CCK and cholinergic agonists have been found to stimulate the release of calcium from the pancreas *in situ* and in vitro, as well as from pancreatic slices and fragments (21). In the present studies we found that CCK-octapeptide and carbamylcholine each stimulated ^{45}Ca outflux from isolated pancreatic acinar cells, and these results provide direct documentation that the effects of CCK and cholinergic agents on calcium transport in intact tissue preparations reflect an action of these secretagogues on the acinar cell. There was no significant effect of either agent on calcium influx. The effect of each of these secretagogues on calcium outflux was rapid and temperature-dependent, and a saturable function of the secretagogue concentration. Stimulation of calcium outflux could be detected within two min after addition of CCK-octapeptide and although basal calcium outflux was not altered by reducing the incubation temperature from 37°C to 4°C, this maneuver abolished the stimulation produced by CCK-octapeptide. Stimulation of calcium outflux was detectable at 5×10^{-11} M CCK-octapeptide, half-maximal at 3×10^{-10} M, and maximal at 5×10^{-9} M. A double-reciprocal plot of stimulation of calcium outflux versus hormone concentration gave a single straight line, indicating that in terms of the biologic response, CCK-octapeptide interacts with a single class of receptors. The effects of

TABLE III

Effect of CCK-Octapeptide and Carbamylcholine on Amylase Release and ^{45}Ca Outflux in Isolated Pancreatic Acinar Cells

	Amylase release	Fractional ^{45}Ca outflux
	%/20 min	*min^{-1}*
Preincubation—10 min		
Control	6.9±1.1	0.06±0.01
CCK-octa (10^{-7} M)	7.2±1.2	0.19±0.02*
Carbamylcholine (10^{-4} M)	7.1±1.0	0.18±0.02*
Preincubation—60 min		
Control	1.3±0.5	0.05±0.01
CCK-octa (10^{-7} M)	4.1±0.7*	0.21±0.02*
Carbamylcholine (10^{-4} M)	3.9±0.6*	0.18±0.02*

Isolated pancreatic acinar cells ($0.5–1 \times 10^6$ cells/ml) were preincubated for the indicated times at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM calcium with or without ^{45}Ca. Cells preincubated without ^{45}Ca were centrifuged (100 *g* for 2 min) and resuspended in fresh buffer containing the indicated agents, and the amylase release was determined during a 20-min incubation at 37°C. For cells preincubated with ^{45}Ca, EDTA (5 mM) plus the indicated agents were added and fractional ^{45}Ca outflux was determined during a 5-min incubation at 37°C. Results shown represent the mean of five separate experiments ±1 SD.

* Significantly greater ($P < 0.01$) than corresponding control by Student's *t* test.

carbamylcholine on calcium outflux were similar to those of CCK-octapeptide in that stimulation of calcium outflux was a saturable function of the secretagogue concentration and carbamylcholine appeared to produce this stimulation by interacting with a single class of receptor sites.

Calcium outflux in isolated pancreatic acinar cells becomes refractory to stimulation by CCK-octapeptide or by carbamylcholine. This effect was not attributable to an effect of incubation per se or to degradation of the secretagogue, since adding fresh CCK-octapeptide or fresh carbamylcholine failed to stimulate calcium outflux in cells previously exposed to the secretagogue but did stimulate control cells incubated for the same duration. Furthermore, stimulation of outflux by CCK-octapeptide or by carbamylcholine produced a refractoriness to both agents. These results indicate that carbamylcholine and CCK-octapeptide affect the same calcium transport mechanism. In addition, stimulation of calcium outflux by maximal concentrations of carbamylcholine plus CCK-octapeptide was the same as that produced by a maximal concentration of either agonist alone.

In intact tissue preparations, atropine inhibits stimulation of amylase secretion and of calcium outflux by cholinergic agents but not that produced by CCK (2, 3). In isolated pancreatic acinar cells, we also found that atropine could abolish stimulation of calcium outflux by carbamylcholine but not that produced by CCK-octapeptide. Atropine, which alone does not alter calcium outflux, is a competitive inhibitor of carbamylcholine, and in pancreatic acinar cells the apparent affinity of the cholinergic receptor for atropine is 10,000 times greater than its apparent affinity for carbamylcholine. The ability of atropine to inhibit the effect of carbamylcholine but not that of CCK-octapeptide indicates that although each of these secretagogues stimulates the same calcium outflux mechanism, the receptors with which they interact to produce this effect are functionally distinct. Furthermore, the apparent affinity of CCK-octapeptide for its receptor was 10^5 greater than that of carbamylcholine for its receptor.

In agreement with the results of Amsterdam and Jamieson (23), we found that CCK-octapeptide and carbamylcholine each stimulated amylase release from pancreatic acinar cells. This stimulation, however, could only be detected in cells preincubated for 60 min. In cells preincubated for 10 min, basal amylase release was elevated and did not increase with CCK-octapeptide or carbamylcholine. In contrast to amylase release, ^{45}Ca outflux as well as the magnitude by which it was stimulated by octapeptide or carbamylcholine did not depend on the duration of preincubation. These results indicate that the source of the ^{45}Ca released from the cells under basal as well as stimulated conditions does not represent release of ^{45}Ca associated with amylase-containing zymogen granules. We have no direct information, however, concerning the source of the ^{45}Ca released from isolated pancreatic acinar cells after exposure to secretagogues. A number of possible sources have been suggested, but not demonstrated, by others (21), and additional studies will be necessary to clarify this issue.

In intact pancreatic tissue preparations, several observations suggest that calcium is involved in stimulation of enzyme release by secretagogues. (*a*) Stimulation of enzyme release by secretagogues in vitro is reduced or abolished in calcium-free media (11, 12, 14, 15, 17, 18). (*b*) Stimulation of ^{45}Ca outflux from pancreas fragments in vitro by cholinergic agents or CCK precedes by a few minutes stimulation of enzyme release (21). (*c*) The relation between amylase release and secretagogue concentration is simlar to that between calcium release and secretagogue concentration (21). (*d*) A calcium-specific ionophore, presumed to accelerate cellular uptake of extracellular calcium, produces stimulation of enzyme secretion equivalent to that produced by cholinergic agents or by CCK (33, 34). Understanding the basis for the difference we observed in the effect of preincubation on amylase release and its lack of effect on ^{45}Ca outflux may further elucidate the role of calcium in pancreatic enzyme secretion.

In contrast to glucagon, secretin, and VIP, but like gastrin, full activity of CCK is shown by a COOH-terminal fragment of the molecule (3, 35, 36). Mutt and Jorpes (3) found that full activity of CCK both on gallbladder contraction and on enzyme secretion from the intact pancreas could be recovered in the COOH-terminal tryptic octapeptide and in the fragments incorporating this octapeptide. Our results comparing the relative potencies of various synthetic analogs and fragments of synthetic COOH-terminal octapeptide of porcine CCK are *qualitatively* similar to those from studies of the relative potencies of analogs and fragments of CCK-octapeptide for stimulating contraction of guinea pig gallbladder (3, 37). Each of the compounds we studied that was capable of producing an effect, at maximal concentrations produced the same stimulation of calcium outflux from pancreatic acinar cells as did a maximally effective concentration of CCK-octapeptide. In terms of their ability to stimulate calcium outflux, the relative potencies of CCK and its fragments were octapeptide > heptapeptide > CCK > hexapeptide = gastrin I = gastrin II = pentagastrin. Others have reported that in addition to the heptapeptide and octapeptide, the COOH-terminal decapeptide and dodecapeptide are more potent than the entire CCK

molecule (3, 37). Thus, the NH_2-terminal portion of the molecule, CCK_{1-21}, reduces the potency of the biologically active COOH-terminal portion, perhaps by sterically hindering the interaction of the COOH-terminal portion with the CCK receptor.

CCK-octapeptide, heptapeptide, CCK, hexapeptide, gastrin, and pentagastrin share a common COOH-terminal tetrapeptide amide that is absolutely required for stimulation of calcium outflux in pancreatic acinar cells. The NH_2-terminal tetrapeptide amide of CCK-octapeptide, CCK_{26-39}, at concentrations as high as 10^{-5} M failed to stimulate calcium outflux; therefore, this portion of the octapeptide molecule, inactive by itself, serves to enhance greatly (by 50,000-fold) the potency of the COOH-terminal tetrapeptide. Additional evidence for the importance of the COOH-terminal tetrapeptide portion of CCK-octapeptide for stimulating calcium outflux is that substituting alanine for the aspartic residue in position 32 reduced its potency by 500-fold and that replacing the methionine residue in position 31 by leucine produced a 100-fold reduction in potency of CCK-octapeptide. CCK-hexapeptide, gastrin I, gastrin II, and pentagastrin were each found not to inhibit CCK-octapeptide stimulation of calcium outflux. This finding appears to exclude the possibility that the COOH-terminal tetrapeptide of CCK-octapeptide is responsible for binding to the receptor and that the remaining amino acids are responsible for producing the biologic effect. Instead, the observed relative potencies appear to reflect the apparent affinities with which the various peptides bind to the CCK receptor on pancreatic acinar cells.

We found that changing the aspartic residue in position 26 to β-aspartic resulted in a compound having the same potency as CCK-heptapeptide. In contrast, in terms of stimulating gallbladder contraction in vitro, the β-aspartic analog was two to four times more potent than CCK-octapeptide (3, 37). BOC-CCK-octapeptide has been reported to be 30-60% less potent than CCK-octapeptide in stimulating gallbladder contraction (3, 37); however, we found this compound to be only 1% as potent as CCK-octapeptide in stimulating calcium outflux from pancreatic acinar cells. While β-aspartic CCK-octapeptide was equipotent with CCK-heptapeptide, BOC-CCK-octapeptide was 10 times less potent than the heptapeptide. These findings suggest that the BOC moiety may introduce a degree of steric hindrance or that a free NH_2-terminal amino group is important for biologic potency.

In other systems a sulfated tyrosine residue has been found to be important but not essential for biologic activity of CCK or CCK-octapeptide (3, 37). In terms of its potency for stimulating calcium outflux in pancreatic acinar cells, desulfated CCK-octapeptide was 1,000 times less potent than the sulfated peptide. We also found that the position of the sulfated tyrosine was important for biologic potency. Exchanging the sulfated tyrosine with the adjacent methionine reduced the potency of CCK-octapeptide by 1,000-fold. This analog has the same COOH-terminal six amino acids as does gastrin II but is 50 times more potent in stimulating calcium outflux. Finally, substituting leucine for both of the methionine residues in CCK-octapeptide results in a compound having the same potency as CCK-octapeptide with only the methionine in position 31 replaced by leucine, suggesting that the methionine in position 28 does not play a major role in determining the potency of CCK-octapeptide.

Secretin, glucagon, and VIP each (at concentrations from 10^{-9} to 10^{-5} M) failed to stimulate calcium outflux from pancreatic acinar cells. In contrast to observations that in vivo secretin can potentiate the stimulation of enzyme release produced by CCK (38-40), we found that secretin did not alter the stimulation of calcium outflux produced by CCK-octapeptide or by carbamylcholine.

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