In Vitro Action of Bombesin and Bombesin-Like Peptides on Amylase Secretion, Calcium Efflux, and Adenylate Cyclase Activity in the Rat Pancreas

A COMPARISON WITH OTHER SECRETAGOGUES

Monique Deschodt-Lanckman, Patrick Robberecht, Philippe De Neef, Marc Lammens, and Jean Christophe

From the Department of Biochemistry and Nutrition, Free University of Brussels Medical School, B-1000 Brussels, Belgium

A b s t r a c t Bombesin (a tetradecapeptide), the C-terminal nonapeptide of bombesin (bombesin-NP), and litorin (a parent nonapeptide), each stimulated amylase secretion from rat pancreatic fragments. These responses were not affected by atropine. The concentrations that produced half-maximal stimulation of secretion were 0.25 nM for bombesin, 0.30 nM for bombesin-NP, and 0.70 nM for litorin, as compared to 0.12 nM for caerulein and 0.80 μM for the cholinergic agent carbamylcholine. When used at maximal concentrations, bombesin, bombesin-NP, and litorin showed no action on cyclic AMP levels in the presence of 5 mM theophylline. By contrast, caerulein and secretin increased cyclic AMP levels by 27 and 208%, respectively. Bombesin, bombesin-NP, and litorin did not activate adenylate cyclase in a purified pancreatic plasma membrane preparation, whereas caerulein and secretin increased this activity 20 and 16-times, respectively.

Calcium efflux was estimated in isolated acinar cells from the rat pancreas. The concentration of bombesin, bombesin-NP, litorin, caerulein, the C-terminal octapeptide of cholecystokinin-pancreozymin, and carbamylcholine that produced half-maximal stimulation of calcium efflux were 0.30 nM, 0.50 nM, 2.00 nM, 0.80 nM, 0.20 nM, and 3.00 μM, respectively. Maximal stimulation of calcium efflux by bombesin or C-terminal octapeptide of cholecystokinin-pancreozymin lasted only 10 min. In addition, refractoriness to a new addition of bombesin was observed after 1 h preincubation with bombesin.

In conclusion, amylase secretion and calcium efflux from the rat pancreas are stimulated in vitro by peptides of the bombesin family.

INTRODUCTION

Until recently, it was believed that the secretion of enzymes by the exocrine pancreas was controlled mainly by two duodenal hormones, secretin and cholecystokinin-pancreozymin (CCK-PZ),[1] and by a muscarinic neurotransmittor, acetylcholine. The discovery of vasoactive intestinal polypeptide (1) and of its specific action on acinar pancreatic cells[2] allowed a first extension of the number of pancreatotropic gastrointestinal hormones. Additional gastrointestinal polypeptides (2) have lately been purified and partially characterized. However, their precise mode of action on the digestive tract in general and on the exocrine pancreas in particular is poorly understood.

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[1] Abbreviations used in this paper: CCK-PZ, cholecystokinin-pancreozymin; CCK-OP, C-terminal octapeptide of CCK-PZ; bombesin-NP, C-terminal nonapeptide of bombesin; Cb, carbamylcholine.

[2] Christophe, J. P., T. P. Conlon, and J. D. Gardner. 1976. Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig: binding of radioiodinated VIP; and Robberecht, P., T. P. Conlon, and J. D. Gardner. 1976. Structural requirements for effects of VIP and secretin on cellular cyclic AMP. J. Biol. Chem. In press.

Recently, peptides of the bombesin family were isolated from the skin of European frogs and shown to exert major effects on extravascular smooth muscle, including gall bladder and intestine. Their interest increased when Erspamer and Melchiorri (3) discovered that cells of the digestive tract contain substances that react with antibombesin antibodies, suggesting that bombesin and its derivatives may also be candidate hormones of the gut (3, 4).

It was therefore of interest to establish the direct action of these peptides on the isolated rat exocrine pancreas. In view of the putative role of calcium movements (5–7) and cyclic AMP (8, 9) in the action of secretagogues on pancreatic secretion, the secretion of amylase induced by these peptides was compared with calcium efflux, cyclic AMP levels, and adenylate cyclase activity.

METHODS

Male Wistar strain albino rats (150–200 g) bred in our laboratory for 15 yr were fed ad libitum on a standard chow (U.A.R. Villemoisson-sur-Orge, France) and killed by cervical fracture or decapitation.

Incubation procedure of pancreas fragments and determination of amylase, cyclic AMP, and proteins. Four pancreases trimmed of fat and major blood vessels were cut into 20–30 mg fragments. Four of these fragments, which weighed 75–100 mg together, were taken at random and shaken in 10-ml beakers containing 2 ml of Krebs-Ringer bicarbonate buffer enriched with 10 mM glucose, Trasylol Bayer, (500 kallikrein inhibitor units U.I.K./ml), and 5 mM theophylline. A pH of 7.4 and oxygenation were maintained under O_2-CO_2 (95 : 5 vol/vol) at 37°C in a metabolic shaker. After a 10-min preincubation period, the medium was discarded and replaced by a fresh medium with the test reagent. The incubation was terminated by pipetting the medium for amylase assay and freezing the fragments over dry ice for cyclic AMP determination.

α-Amylase in the medium was estimated by the saccharogenic method of Noelting and Bernfeld (10) as automated by Vandermeers et al. (11). The unit of amylase is defined as the amount of enzyme that liberates a reducing power equivalent to 1 μmol of maltose per min at 25°C. Tissue protein was determined according to the method of Lowry et al. (12) by using bovine albumin as a standard. Cyclic AMP was determined by the protein binding assay of Gilman (13).

Isolation of rat pancreatic acinar cells and investigation of ^{45}*Ca outflux.* Isolated pancreatic acinar cells were prepared from four to six rats by the procedure of Amsterdam and Jamieson (14) by using crude collagenase and hyaluronidase, EDTA, and mild shearing forces. The digestion of the rat pancreas and the isolation of acinar cells offered no more difficulty than that of the guinea pig. The three cell washes ending the procedure were conducted at a 0.1-mM calcium concentration. These cells were finally resuspended (5–9 × 10⁶ cells/ml) in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.5 mM ^{45}Ca, 1% (wt/vol) bovine serum albumin, 14 mM glucose, and Trasylol (500 U.I.K./ml). At appropriate time intervals cellular ^{45}Ca was measured on duplicate 100-μl samples as described by Gardner et al. (5). In brief, cells contained in each sample were washed by three centrifugations (15 s at 10,000 g) with a Beckman model 153 microfuge (Beckman Instru-

ments, Inc., Spinco Div., Palo Alto, Calif.) in a Krebs-Ringer bicarbonate buffer (pH 7.4), enriched with 2.5 mM Ca, and 1% (wt/vol) albumin.

Peptides, reagents, and chemicals. Bovine plasma albumin, fraction V (fatty acid poor), was purchased from Miles Laboratories, Inc., Elkhart, Ind.) ; chromatographically purified soybean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, N. J.; crude bovine-testis hyaluronidase and crude collagenase were from Sigma Chemical Co., St. Louis, Mo. Trasylol was a gift from Bayer-Pharma, Brussels, Belgium. Bombesin (TP/18998), the C-terminal nonapeptide of bombesin (Bombesin-NP TF/18831) and litorin (TF/9207) were generously provided by Dr. R. de Castiglione (Farmitalia S.p.A. Laboratories for Basic Research, Milan, Italy). The C-terminal octapeptide of CCK-PZ (CCK-OP) was a gift from Dr. Miguel Ondetti (Squibb Institute for Medical Research, Princeton, N. J.). The natural porcine secretin was purchased from the Gastrointestinal Hormone Research Unit of the Karolinska Institutet, Stockholm, Sweden. The radioactive products were purchased from the Radiochemical Centre, Amersham, England. The specific radioactivity for [8-³H]-cyclic AMP, and ^{45}Ca were respectively, 27 mCi/mmol, and 10–40 mCi/mg. All other reagents and chemicals were of the highest grade available.

RESULTS

Effect of bombesin and bombesin-like peptides on amylase secretion. Bombesin, bombesin-NP, and litorin were powerful stimulants of amylase secretion from rat pancreatic fragments. Fig. 1A shows that three to six fold increases were induced by 1 nM bombesin within 5 min and were maintained for at least 30 min. The time
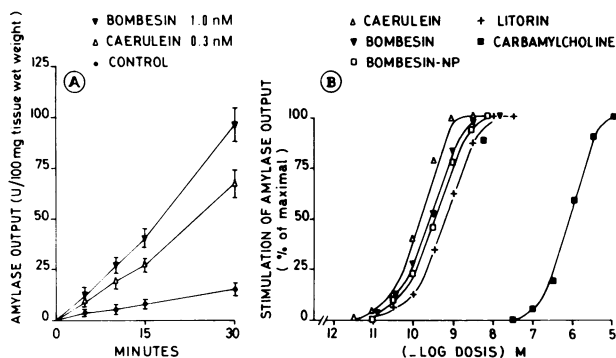

FIGURE 1 Effect of bombesin and bombesin-like peptides on amylase secretion from rat pancreatic fragments.

1A : Time course of action of bombesin and caerulein. Results are expressed in amylase U/100 mg tissue. The control value (●——●) are the means of eight experiments. The values obtained in presence of 1 nM bombesin (▼——▼) or 0.3 nM caerulein (△——△) are the means of four experiments. Vertical bars represent SEM.

1B : Dose-response curve for amylase release in response to caerulein (△——△), bombesin (▼——▼), bombesin nonapeptide (□——□), litorin (+ —— +), and carbamylcholine (■——■). Each curve represents the mean of four experiments. Amylase output was determined in the medium after 15 min incubation as described under Methods and the results are expressed as % of maximum stimulation.

study of this effect was similar to that obtained with 0.3 nM caerulein.

Figure 1B illustrates dose-response curves for amylase output in response to bombesin, bombesin-NP, litorin, caerulein, and carbamylcholine. Inasmuch as these peptides had the same maximal effects, results were expressed in percent of the maximal response. Molar concentrations of bombesin, bombesin-NP, litorin, caerulein, and carbamylcholine provoking 50% of this effect were 0.25, 0.30, 0.70, and 0.12 nM, and 0.80 μM, respectively. These results were obtained in the presence of 5 mM theophylline, to amplify rises in cyclic AMP occurring in the same fragments (9) (Table I). However, theophylline per se had no effect on amylase secretion under the present conditions (9). Finally, the action of bombesin and its analogs on amylase secretion was not abolished by atropine at a concentration that inhibited almost completely a secretion induced by a maximal concentration of carbamylcholine (data not shown).

Effects of bombesin and bombesin-like peptides on pancreatic cyclic AMP levels and adenylate cyclase activity. Table I indicates the effect of different secretagogues, used alone or in combination, on cyclic AMP levels tested after 15 min incubation in the presence of 5 mM theophylline. 0.1 μM secretin induced a threefold increase whereas 1 μM caerulein provoked a modest but significant 27% rise in cyclic AMP. Bombesin and related peptides did not influence the levels of cyclic AMP. Even concentrations as high as 10 μM were ineffective (data not shown). On the other hand, bombesin, bombesin analogs, and caerulein all significantly inhibited the rise of cyclic AMP induced by secretin (Table I).

The effects of bombesin and bombesin-like peptides were tested directly on the hormone-sensitive adenylate cyclase of purified pancreatic plasma membranes.[3] These peptides were unable to stimulate such preparations, even when used at concentrations as high as 10 μM. In contrast, these membranes were highly responsive to secretin and caerulein (16-fold and 20-fold stimulations, respectively, data not shown).

Effect of bombesin and bombesin-like peptides on ^{45}Ca *movements.* Uptake of the tracer by isolated rat pancreatic acinar cells progressed relatively quickly at first but did not increase much after 45 min of incubation (Figs. 2 and 4). ^{45}Ca outflux was therefore tested in this and in subsequent experiments on cells that had been preincubated with ^{45}Ca for at least 50 min.

After addition of 10 nM bombesin or 10 nM CCK-OP, to cells that had been preincubated with the tracer for

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[3] Details on the purification of a stable and hormone-responsive rat pancreatic adenylate cyclase, and properties of this semipurified plasma membrane fraction will be given in another paper (Svoboda, M., P. Robberecht, J. Camus, M. Deschodt-Lanckman, and J. Christophe. Manuscript in preparation).

Additions			Cyclic AMP
Control			3.7±0.3
Secretin	100 nM		11.4±1.0*
Caerulein	10 nM		3.4±0.4
	1 μM		4.7±0.5*
Bombesin	100 nM		3.2±0.2
Bombesin-NP	100 nM		3.5±0.3
Litorin	100 nM		3.8±0.2
Secretin	100 nM + Caerulein	10 nM	8.5±0.4‡
	100 nM + Caerulein	1 μM	8.5±0.4‡
	100 nM + Bombesin	1 nM	10.1±0.5
	100 nM + Bombesin	100 nM	8.6±0.5‡
	100 nM + Bombesin-NP	1 nM	10.8±0.5
	100 nM + Bombesin-NP	100 nM	9.7±0.3‡
	100 nM + Litorin	1 nM	10.6±0.2
	100 nM + Litorin	100 nM	9.5±0.4‡

Cyclic AMP was determined after 15 min incubation in the presence of 5 mM theophylline as described under Methods. Results in pmol/mg tissue protein are the means±SEM of seven experiments.
* Cyclic AMP levels significantly greater ($P < 0.05$) than values observed with controls, when employing Student's t test for paired data comparison.
‡ Cyclic AMP levels significantly lower ($P < 0.05$) than values observed with secretin alone.

50 min, cellular ^{45}Ca declined swiftly to approximately half of control levels after 5–10 min and fell more slowly during the succeeding 40 min (Fig. 2).

Introduction of 5 mM EDTA, which chelates extracellular calcium and prevents influx, induced a 40% decrease in cellular ^{45}Ca that echoed calcium outflux. Exposing the isolated pancreatic acinar cells to 10 nM bombesin plus 5 mM EDTA, or 10 nM CCK-OP plus 5 mM EDTA, accelerated the loss of ^{45}Ca, implying that both peptides were depressing cellular ^{45}Ca by stimulating calcium outflux.

Extending findings previously reported by Gardner et al. (5) on guinea pig acinar cells to the rat, we were able to show that a semilog plot of the loss of cellular ^{45}Ca with EDTA only or with bombesin or CCK-OP, determined at 1-min intervals, was linear for 6 min, after which the slope decreased (data not shown and Fig. 2). Consequently, calcium efflux was acceptably estimated from the loss of ^{45}Ca during a 4-min incubation with 5 mM EDTA.

The addition of 10 nM bombesin or 10 nM CCK-OP produced a twofold stimulation of fractional calcium efflux (17.3±2.6 and 17.8±2.1%/min, respectively, as compared to 8.6±2.6%/min for control values; means ±1 SD of eight separate experiments).

• CONTROL
o CCK-OP 10 nM
■ BOMBESIN 10 nM
□ EDTA 5 mM
△ EDTA + CCK-OP
▲ EDTA + BOMBESIN
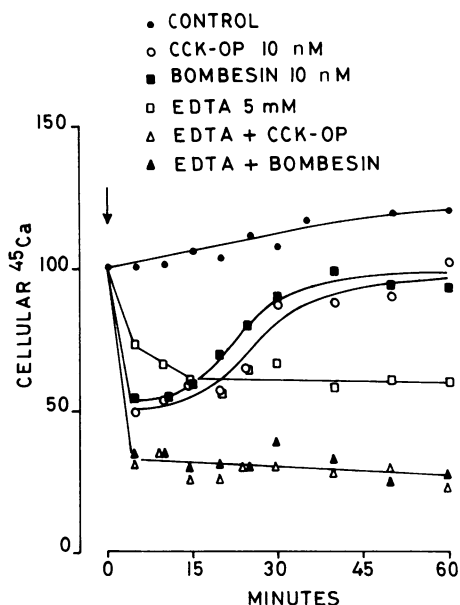
FIGURE 2 Effect of 10 nM bombesin and of 10 nM CCK-OP on cellular ^{45}Ca in rat pancreatic acinar cells. Isolated cells were preincubated with 0.5 mM ^{45}Ca at 37°C as described under Methods. After 50 min, 5 mM EDTA and/or bombesin and/or CCK-OP was added (↓) and cellular ^{45}Ca determined at intervals during a subsequent 60-min incubation. Cellular ^{45}Ca is given as a percentage of the value observed at time 50 min. Each point is the mean of duplicate determinations and this experiment is representative of two others.

The twofold stimulation of the rate of calcium loss produced by maximal concentrations of bombesin plus CCK-OP or carbamylcholine was the same as that produced by a maximal concentration of either agent alone, i.e. stimulation of calcium efflux by bombesin overlapped completely with that produced by CCK-OP or carbamylcholine. The cholinergic antagonist atropine (40 μM) did not alter basal calcium outflux in isolated pancreatic acinar cells or the stimulation of calcium efflux produced by bombesin but abolished the effect of carbamylcholine (data not shown).

Stimulation of calcium efflux was a saturable function of the concentration of bombesin, CCK-OP, and caerulein. The concentrations that produced half-maximal stimulation of ^{45}Ca efflux were 0.30 nM for bombesin, 0.20 nM for CCK-OP, and 0.80 nM for caerulein, respectively (Fig. 3).

The chemical specificity needed for stimulation of calcium efflux by peptides of the bombesin family was partially explored. Of the three peptides tested, the tetradecapeptide bombesin was the most potent. Bombesin-NP was almost as potent as the complete bombesin molecule: removing the five first residues from bombesin lowered its potency by only 40% (Fig. 3). The potency of litorin was only 15% of that of bombesin (Fig. 3).

The cholinergic agent carbamylcholine also stimulated calcium outflux from isolated pancreatic acinar cells, and the concentration which produced half-maximal stimulation was 3 μM (Fig. 3).

Fig. 4 illustrates two time studies of the stimulation of calcium efflux in acinar cells exposed to 10 nM bombesin or 10 nM CCK-OP. The transient decrease in ^{45}Ca content after the addition of both peptides was similar to that observed in Fig. 2A. In addition, it is clear that the concurrent 2.5-fold stimulation of calcium outflux was short-lived (Fig. 2B). This stimulation declined with time in both cases; it remained significant only after CCK-OP treatment whereas calcium outflux was essentially back to normal within 10 min of bombesin addition.

Isolated pancreatic acinar cells that had been preincubated with 10 nM bombesin for 50–60 min failed to show stimulation of calcium efflux with a second addition of bombesin but were able to respond to 10 nM CCK-OP (Table II). On the other hand, preincubating isolated pancreatic cells with 10 nM CCK-OP abolished the stimulation of calcium outflux produced by bombesin or by CCK-OP.

DISCUSSION

Caerulein was the first peptide isolated from the skin of an amphibian (the Australian hylid frog Hyla caerulea)
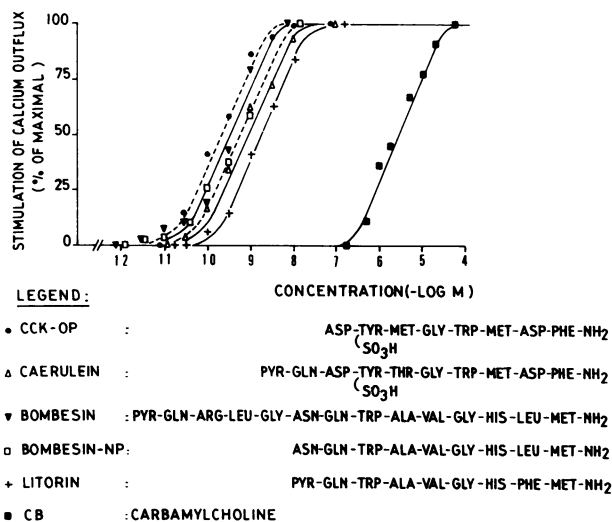

LEGEND:

• CCK-OP : ASP-TYR-MET-GLY-TRP-MET-ASP-PHE-NH$_2$
                 (SO$_3$H

△ CAERULEIN : PYR-GLN-ASP-TYR-THR-GLY-TRP-MET-ASP-PHE-NH$_2$
                 (SO$_3$H

▼ BOMBESIN : PYR-GLN-ARG-LEU-GLY-ASN-GLN-TRP-ALA-VAL-GLY-HIS-LEU-MET-NH$_2$

□ BOMBESIN-NP: ASN-GLN-TRP-ALA-VAL-GLY-HIS-LEU-MET-NH$_2$

+ LITORIN : PYR-GLN-TRP-ALA-VAL-GLY-HIS-PHE-MET-NH$_2$

■ CB : CARBAMYLCHOLINE

FIGURE 3 Dose-effect relationship of bombesin (▼——▼), bombesin nonapeptide (□--□), litorin (+——+), caerulein (△——△), CCK-OP (●--●), and carbamylcholine (CB: ■——■) on calcium outflux. Isolated rat acinar cells were preincubated at 37°C with 0.5 mM ^{45}Ca for 50–70 min as described under Methods. Calcium outflux was determined from the loss of cellular ^{45}Ca during a 4-min incubation after addition of 5 mM EDTA and calculated as the fractional rate of loss per min. Stimulation of calcium outflux is given as the fraction of maximal stimulation. Each point was determined in duplicate and results shown are means of 6–10 separate experiments.
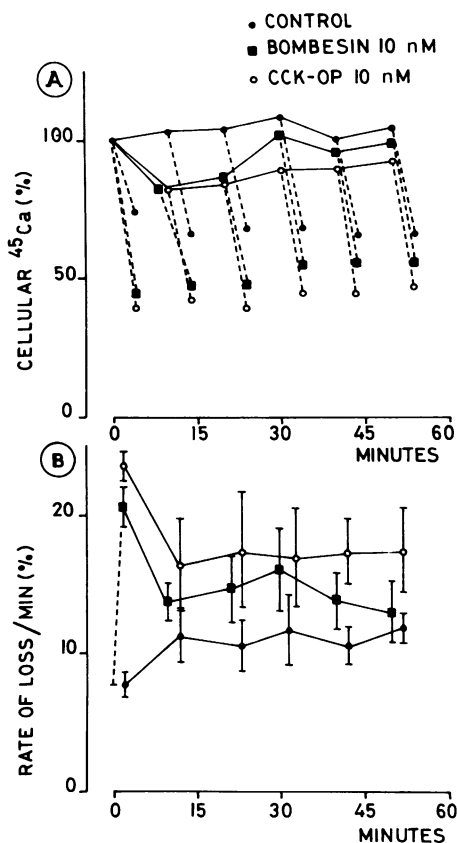
FIGURE 4 Time study of the stimulation of calcium outflux from rat pancreatic acinar cells exposed to 10 nM bombesin (■——■) or 10 nM CCK-OP (O——O). Isolated cells were preincubated at 37°C with 0.5 mM ^{45}Ca as described under Methods. After 60 min, three series of aliquots were organized. Bombesin was added to the second series (■——■) and CCK-OP to the third series (O——O). Cellular ^{45}Ca was determined at 10-min intervals during a subsequent 50-min incubation before and 4-min after the addition of 5 mM EDTA (---). Each point was determined in duplicate and results shown are means of five separate experiments.

4A: Cellular ^{45}Ca is given as a percentage of the value observed at time 60 min of the preincubation.

4B: The time study of calcium outflux was estimated from data in Fig. 4A and expressed as average fractional rates of loss/min (percent) ±1 SD ($n = 5$) observed at 10-min intervals.

and was found to be closely related to a gastrointestinal hormone, CCK-PZ. More recently, bombesin, a tetradecapeptide, and litorin, a nonapeptide, were extracted, respectively, from the skin of two European amphibians of the family Discoglossidae (*Bombina bombina* and *Bombina variegata variegata*) and from the skin of an Australian leptodactylid frog (*Litoria aurea*). These active peptides are similar and have been synthesized. They differ totally from the CCK-PZ family including caerulein.

Fresh interest in peptides of the bombesin family has resulted from the discovery that bombesin-like peptides are present in the antral and duodenal mucosa of dogs and pigs (3) as shown by radioimmunoassay.

The Italian investigators have recently demonstrated that a perfusion of bombesin markedly stimulates the secretion of pancreatic juice rich in hydrolases in man (15), dog (16), and chicken (17). Erspamer et al. (16) have suggested that the pancreatic response and the acceleration of gall bladder emptying (16, 18) are secondary to the release of CCK-PZ from the duodenal mucosa. However, they recognize that such a mechanism is only presumptive and that direct evidence will be lacking until a reliable radioimmunoassay of blood CCK-PZ is feasible. By contrast, increased serum gastrin levels have been unequivocally demonstrated in the dog in response to bombesin infusion (19, 20).

Compared with the whole animals, where interactions between organs are unavoidable, in vitro systems are more convenient for a demonstration of direct secretory effects of bombesin on rat pancreatic acinar cells. The in vitro system is ideally suited for a simultaneous determination of amylase output and cyclic AMP levels: the linear secretory response (Fig. 1A) can be correlated directly with the plateau of cyclic AMP levels attained after 15 min whenever the adenylate cyclase system was stimulated (9). A study of the dose-response curves of calcium efflux on the same model was not feasible in view of the rapidity of cellular ^{45}Ca kinetics and the time that would be required for a discharge of

TABLE II
Refractoriness of Calcium Outflux in Isolated Rat Pancreatic Acinar Cells

Preincubation	Added during incubation	Calcium efflux
50–60 min		*%/min*
None	None	9.4±1.8
None	Bombesin	23.2±1.3*
None	CCK-OP	23.4±1.3*
Bombesin	None	9.6±1.8
Bombesin	Bombesin	8.2±1.7
Bombesin	CCK-OP	19.9±0.9*
CCK-OP	None	8.6±1.3
CCK-OP	Bombesin	7.9±1.2
CCK-OP	CCK-OP	9.9±1.9

Isolated rat pancreatic acinar cells were preincubated at 37°C for 50–60 min with 0.5 mM ^{45}Ca plus the indicated agents as described under Methods. Calcium outflux was determined from the loss of cellular radioactivity during a 4-min incubation with 5 mM EDTA plus addition of the agents indicated. The concentrations of bombesin and CCK-OP were 10 nM. Results given are the means of 10 separate experiments ±1 SD.
* Significantly greater ($P < 0.05$) than calcium outflux with no additions using Student's t test.

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[45]Ca from the extracellular space of fragments of irregular thickness.

It is theoretically conceivable that bombesin was in fact stimulating endocrine cells, intermingled with the exocrine tissue, to release hormones of the gastrin CCK-PZ family and acinar cells to increase amylase secretion in response to these hormones. Immunoreactive gastrin has indeed been measured by Thompson et al. in the rat pancreas (21). The release to the medium of the total content reported to be present (2 μg/g wet weight) would allow a 50 nM gastrin concentration under our incubation conditions. However, gastrin is only 0.1% as efficient as caerulein on hydrolase output from rat pancreas fragments (unpublished data from our laboratory). This hormone is thus relatively less potent in our system than in dog (22, 23), and a 50-nM concentration of endogenous gastrin could not induce more than 20% of the maximal stimulation of amylase secretion observed with bombesin. The presence in the rat pancreas of cells containing pancreozymin-like material has never been reported to the best of our knowledge. This possibility cannot be ruled out but would not explain why after 1 h preincubation with bombesin refractoriness to a new addition of bombesin was observed, whereas isolated cells were still able to respond to CCK-OP in terms of calcium outflux (Table II and *vide infra*). This could not occur if the effect of bombesin was mediated by the release of CCK-PZ. In this connection, we must admit that if the contribution of endocrine cells secreting hormones of the pancreozymin-gastrin family is, at best, dubious in pancreas fragments, then the active presence of such cells in dispersed acinar cell preparations was even more unlikely because of the enrichment in acinar cells occurring during the isolation procedure. By light microscopy, at least 98% of such supensions were made of acinar cells, and contamination by erythrocytes and centroacinar cells was minimal. In addition, microscopic examination also revealed that more than 98% of the cells were able to exclude trypan blue, suggesting good viability.

It is thus reasonable to consider that most, if not all, the effects of bombesin on our pancreatic preparations were due to a direct action on acinar cells and that bombesin was at least as efficient on a molar basis as the other already well documented frog peptide caerulein (Figs. 1B and 3).

Our parallel bioassays of bombesin, bombesin-NP, and litorin on calcium outflux (Fig. 3) and amylase secretion (Fig. 1B) confirm that the C-terminal nonapeptide was sufficient for maximal biological activity. In addition, substituting the asparaginyl C-terminal residue in bombesin-NP by pyroglutamyl and the leucyl[8] residue by phenylalanyl[8] resulted in a compound (litorin) whose potency was three to seven times lower than that of bombesin-NP. This could be due to a lower affinity for receptor sites or to more rapid degradation. Similar relative potency for bombesin, bombesin-NP, and litorin have been reported in vivo in the dog (24, 25).

The mechanism of the direct action of bombesin on acinar cells has been partially elucidated. Bombesin appears to act primarily on calcium efflux (Figs. 3 and 4).

At this stage, it must be noted that the basal efflux of calcium from isolated pancreatic acinar cells was higher in the rat (8.6%/min) than in the guinea pig (4.3%/min) (5). In addition the dose-effect relationship for CCK-OP suggests a somewhat greater sensitivity of rat acinar cells (Fig. 3). We cannot state whether these qualitative differences between rat and guinea pig reflect real differences between the two species or another factor such as the batch of collagenase or hyaluronidase used for isolating acinar cells. At any rate, most data were similar and the maximal outflux of calcium observed after addition of a number of peptides or carbamylcholine was comparable in both species (17-18%/min).

A maximally effective concentration of bombesin produced the same stimulation of calcium efflux and amylase secretion as did a maximal concentration of CCK-OP or carbamylcholine. In addition, no additive effects were observed on these parameters when bombesin and the other secretagogues were offered in combination at maximal concentration. Furthermore, the concentrations of bombesin and other secretagogues that produced half-maximal stimulation of calcium outflux (Fig. 3) were approximately the same as those provoking half-maximal stimulation of secretion (Fig. 1B), in spite of the added steps required for the dispersion and preincubation of acinar cells in the study of [45]Ca outflux.

The stimulation of calcium efflux after a single addition of bombesin was short-lived (Fig. 4), whereas the in vitro secretory effect was longer (Fig. 1A) and continued for at least 2 h (data not shown). The difference in the time-course of calcium efflux and amylase secretion also was exemplified by the finding that acinar cells previously exposed to bombesin failed to increase calcium outflux again when fresh bombesin was added 1 h later (Table II). The refractoriness of calcium efflux to CCK-OP proved to be similar to that already observed by Gardner et al. (5) with acinar cells from the guinea pig. Additional studies are necessary to unravel the underlying mechanism, but it is already clear that the effects of bombesin on calcium efflux were basically similar to those exerted by peptides of the pancreozymin family and by carbamylcholine. It is also worth noting that none of these agents were able to affect calcium accumulation. Steady-state levels of radioactivity remained similar to control values (data not shown), a finding in apparent contradiction with that of Kondo and Schultz (26) who reported that CCK-OP and carbamylcholine stimulate [45]Ca accumulation in isolated

rat pancreas cells. This discrepancy might be partially due to differences in calcium concentration and washing procedure.

Stimulus-secretion coupling in the exocrine pancreas involves not only calcium (5–7, 26) but also cyclic GMP (27–29) and cyclic AMP (8, 9, 29, 30) in vivo and in vitro. The transitory increases in cyclic GMP can be correlated with calcium movements. Rises in cyclic AMP levels may be an alternative mechanism for stimulating hydrolase secretion. Indeed, secretin induces a sustained, dose-related secretion of amylase in rat (9, 31) and guinea-pig (Gardner, personal communication). This hormone binds to specific receptors coupled to adenylate cyclase subunits in dispersed acinar cells [2] (30) but exerts no significant effect on calcium movements in the same preparation (5). Thus, our data on cyclic AMP levels and adenylate cyclase activity clearly exclude the possibility that bombesin stimulates hydrolase secretion by a secretin-like mechanism. However, high concentrations of peptides of the bombesin family were able to inhibit the rise of cyclic AMP induced by secretin. Similar moderate (-15 to -25%) but significant reductions were also induced by caerulein (Table I) and the divalent cation ionophore A-23187 (29). It is tempting to suggest that these effects were secondary to shifts in cellular calcium (29), considering that free calcium inhibits adenylate cyclase in a number of systems (32).

The mechanism of action of bombesin was not cholinergically dependent since bombesin effects were not inhibited by atropine. It may also be distinguished from caerulein action since bombesin did not stimulate adenylate cyclase in pancreatic plasma membrane preparations. In addition we have observed that bombesin does not compete with [³H] caerulein for binding to isolated acinar cells and purified plasma membranes.[4]

In conclusion, bombesin must be added to the list of secretagogues stimulating hydrolase secretion and calcium outflux in pancreatic acinar cells.

ACKNOWLEDGMENTS

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