Action of Cholecystokinin, Cholinergic Agents, and A-23187 on Accumulation of Guanosine 3':5'-Monophosphate in Dispersed Guinea Pig Pancreatic Acinar Cells

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The COOH-terminal octapeptide of cholecystokinin (CCK-OP) and carbamylcholine each increased calcium outflux, cellular cyclic GMP and amylase secretion in dispersed guinea pig pancreatic acinar cells. Following addition of CCK-OP or carbamylcholine, cellular cyclic GMP increased as early as 15 s, became maximal after 1 to 2 min, and then decreased steadily during the subsequent incubation. For both CCK-OP and carbamylcholine there was close agreement between the dose-response curve for stimulation of calcium outflux and that for increase of cellular cyclic GMP. With CCK-OP an effect on both functions could be detected at 10⁻⁸ M and maximal stimulation occurred at 3 x 10⁻⁸ M. With carbamylcholine an effect on both functions could be detected at 10⁻⁵ M and maximal stimulation occurred at 3 x 10⁻⁴ M. Atropine inhibited stimulation of both cyclic GMP and calcium outflux by carbamylcholine but not by CCK-OP. Stimulation of calcium outflux or cellular cyclic GMP by CCK-OP or carbamylcholine did not require extracellular calcium since stimulation occurred in a calcium-free, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA)-containing solution.

The divalent cation ionophore A-23187 increased bidirectional fluxes of calcium, cellular cyclic GMP and secretion of amylase from dispersed pancreatic acinar cells. Like CCK-OP and carbamylcholine, the ionophore stimulated calcium outflux and cellular cyclic GMP in a calcium-free, EGTA-containing solution. These results suggest that in pancreatic acinar cells the initial step in the sequence of events mediating the action of ionophore as well as that of CCK-OP and carbamylcholine is stimulation of calcium outflux, and that this stimulation then increases cellular cyclic GMP.

Cholecystokinin and related peptides, as well as muscarinic cholinergic agents, stimulate enzyme secretion (1-3) and calcium release (4-6) from the pancreas in vitro. More recently we (7) have found that these same effects can be detected using a homogeneous suspension of acinar cells prepared from guinea pig pancreas. Since muscarinic cholinergic agents have been found to increase cyclic GMP¹ in several different tissues (8), and since intravenous injection of cholecystokinin or pilocarpine produces a transient doubling of cyclic GMP in rat pancreas in vivo (9), we have explored the effect of CCK-OP and related peptides as well as the muscarinic cholinergic agent carbamylcholine on accumulation of cyclic GMP in dispersed pancreatic acinar cells. We have also compared effects of a particular agent on cyclic GMP accumulation to effects of that same agent on outflux of "Ca from pancreatic acinar cells. Finally, to better understand the relation between stimulation of calcium outflux and stimulation of cellular cyclic GMP, we have tested the effects of the divalent cation ionophore, A-23187 (10), on these two processes.

MATERIALS AND METHODS

Male Hartley albino guinea pigs (350 to 400 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md. Synthetic COOH-terminal octapeptide of porcine cholecystokinin (CCK-OP; SQ 19,844), COOH-terminal heptapeptide of porcine cholecystokinin (SQ 19,244), and desulfated COOH-terminal octapeptide of cholecystokinin (SQ 19,265) were
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Results

In dispersed pancreatic acinar cells which had been preloaded with 46Ca and then incubated with CCK-OP (10-7 M) cellular 46Ca decreased rapidly to 50% of control after 4 min and remained at this level for the duration of the 10-min incubation (Fig. 1). Coincident with this change in cellular 46Ca, cyclic GMP increased as early as 15 s, was maximal (11 fold increase) after 1 min, and then decreased steadily toward control values during the remainder of the incubation (Fig. 1). Cholecystokinin has also been found to increase cyclic GMP in rat pancreas in vivo (9) and in rabbit gall bladder in vitro (18). Carbacholcholine (10-5 M) which increases cyclic GMP in gastrointestinal, as well as other tissues (8), produced changes in cellular 4Ca and cyclic GMP which were identical to those caused by CCK-OP. Secretin (10-6 M) which under

We have shown previously (7) that if the incubation is continued beyond 10 min cellular 4Ca begins to increase after 15 to 20 min and returns to control values by 60 min.

Fig. 1. Effect of CCK-OP on cyclic GMP and 4Ca in dispersed pancreatic acinar cells. Cells were preincubated for 40 min at 37° in standard incubation solution containing 0.5 mM calcium with or without 4Ca. In cells preincubated without 4Ca, duplicate samples were taken to determine basal cyclic GMP, CCK-OP (10-7 M) was added, and cyclic GMP was determined at various times during a 10-min incubation at 37°. In cells preincubated with 4Ca, duplicate samples were taken to determine basal cellular 4Ca, CCK-OP (10-7 M) was added, and cellular 4Ca was determined at various times during a 10-min incubation at 37°. Results for cellular 4Ca and cyclic GMP are expressed as the fraction of the amount determined in basal samples.

These same conditions produced a 10-fold increase in cyclic AMP (19, 20), did not alter cyclic GMP or cellular 4Ca. When this same experiment was performed without theophylline in the incubation medium, the changes in cellular 4Ca were unaltered but there was no detectable increase in cellular cyclic GMP (results not shown). In the pancreas we (19, 20) and others (21) have also found that adding theophylline does not alter basal cyclic AMP but is essential to detect increased cyclic AMP produced by a particular agent.

There was good agreement between the dose-response curve for CCK-OP-stimulated 4Ca outflux and that for increased cyclic GMP (Fig. 2). For both processes significant stimulation could be detected by 10-16 M CCK-OP and maximal stimulation occurred at 3 x 10-7 M. Close agreement was also obtained with the COOH-terminal heptapeptide of cholecystokinin which we have previously shown to be one-tenth as potent as CCK-OP in stimulating 4Ca outflux (7) and which in the present studies was one-tenth as potent as CCK-OP in increasing cyclic GMP (Fig. 2). Using desulfated CCK-OP which is 1000 times less potent than CCK-OP (7) we also found close agreement between the dose-response curve for stimulation of 4Ca outflux and that for stimulation of cellular cyclic GMP. Secretin, at concentrations as high as 10-4 M, failed to increase 4Ca outflux or cellular cyclic GMP (Fig. 2). Like the COOH-terminal fragments and analog of cholecystokinin, there was also good agreement between the dose-response curve for carbamylcholine-stimulated 4Ca outflux and carbamylcholine-stimulated cyclic GMP (Fig. 2). For both processes significant increases could be detected at 10-5 M carbamylcholine and maximal stimulation occurred at 3 x 10-5 M. Results similar to those given in Fig. 2 were obtained by measuring net cellular release of 4Ca (i.e., the decrease in cellular 4Ca after a 5-min incubation with the indicated agents but without EDTA) instead of 4Ca outflux (i.e., the decrease in

4Ca fluxes in dispersed pancreatic acinar cells are not altered by 2 mM theophylline. Furthermore, the magnitude of the effects of peptides, carbamylcholine or A-23187 on 4Ca fluxes is not altered by 2 mM theophylline.
CONCENTRATION (M)

FIG. 2. Effect of secretin, carbamylcholine and three synthetic COOH-terminal fragments of cholecystokinin on cyclic GMP and on \(^{45}\)Ca outflux in dispersed pancreatic acinar cells. To determine cyclic GMP, cells were preincubated for 5 min at 37° in standard incubation solution. The indicated concentration of secretin, carbamylcholine, or COOH-terminal octapeptide, heptapeptide or desulfated octapeptide of cholecystokinin was added and cyclic GMP was determined after 2 min of incubation at 37°. Results are expressed as the percentage of stimulation of cyclic GMP produced by 10^{-6} M CCK-OP. To determine cellular \(^{45}\)Ca after a 5-min incubation with the indicated agents plus EDTA).

As we have observed previously, studying \(^{45}\)Ca transport in pancreatic acinar cells (7), that the muscarinic cholinergic antagonist atropine produced a concentration-dependent inhibition of the increase in cellular cyclic GMP produced by 10^{-3} carbamylcholine but did not alter the increase produced by 10^{-9} M CCK-OP (Fig. 3). Significant inhibition of carbamylcholine-stimulated cyclic GMP could be detected at 10^{-9} M atropine and the effect of carbamylcholine was abolished by 10^{-7} M atropine. The magnitude of the increase in cellular cyclic GMP with maximal concentrations of CCK-OP plus carbamylcholine was the same as that produced by a maximal concentration of either agent alone (results not shown).

Neither CCK-OP (10^{-7} M) nor carbamylcholine (10^{-3} M) altered uptake of \(^{45}\)Ca by dispersed pancreatic acinar cells during the initial 15 min of the uptake process (Fig. 4A). Cells which were preloaded with 0.5 mM \(^{45}\)Ca and then incubated with 5 mM EDTA (to chelate extracellular calcium and thereby abolish calcium influx) lost 20% of their \(^{45}\)Ca during the first 5 min of incubation and approximately 40% during a 15-min incubation (Fig. 4B). Adding 5 mM EDTA plus 10^{-6} M CCK OP or 10^{-3} M carbamylcholine significantly increased the rate of loss of \(^{45}\)Ca (Fig. 4B). In contrast to CCK-OP and to carbamylcholine, the divalent cation ionophore A-23187 produced a 4- to 5-fold increase in uptake of \(^{45}\)Ca (Fig. 4A). In cells which had been preloaded with 0.5 mM \(^{45}\)Ca and were then incubated with 5 mM EDTA, the ionophore also increased \(^{45}\)Ca outflux, and this increase was greater than that obtained with maximal concentrations of CCK-OP or carbamylcholine (Fig. 4B). Since A-23187 increased bidirectional fluxes of \(^{45}\)Ca in dispersed pancreatic acinar cells, and since the ionophore has been found to stimulate amylase secretion from slices of pancreas in vitro (22, 23), we tested its effect on amylase secretion from dispersed pancreatic acinar cells and found that, like CCK-OP and carbamylcholine, A-23187 (5 x 10^{-6} M) produced a 2- to 3-fold stimulation of amylase release. Atropine did not alter basal enzyme release or that stimulated by CCK-OP or A-23187 but abolished the increase produced by carbamylcholine. Like CCK-OP and carbamylcholine, A-23187 also stimulated
cellular cyclic GMP in dispersed pancreatic acinar cells (Fig. 5). Adding A-23187 increased cyclic GMP to values which were 14 times basal after 2 min of incubation. Cellular cyclic GMP then fell progressively during the subsequent 12 min of incubation. This experiment was then repeated using cells which were suspended in an incubation medium which was free of calcium and contained 0.2 mM EGTA (Fig. 5). Removing extracellular calcium and adding EGTA did not alter basal values for cyclic GMP. Adding A-23187 to cells in a calcium-free medium increased cyclic GMP in a manner similar to that obtained when calcium was present in the incubation. Similar results were obtained using EDTA instead of EGTA.

To see whether extracellular calcium affected the ability of CCK-OP to increase cyclic GMP, dispersed pancreatic acinar cells were suspended in solutions containing different concentrations of calcium and cyclic GMP was measured at the end of a 2- or 3-min incubation with CCK-OP. The increase in cyclic GMP produced by CCK-OP was not dependent on extracellular calcium concentrations from 0 through 2.5 mM (Fig. 6). Similar results were obtained with carbamylcholine (10^{-3} M) and with A-23187 (5 \times 10^{-6} M).

**DISCUSSION**

In the present study using dispersed pancreatic acinar cells we found a good correlation between the ability of a particular agent to increase cellular cyclic GMP and its ability to stimulate calcium outflux. An increase in both functions could be detected as early as it was technically feasible to take samples, i.e., 1 min for calcium outflux and 15 s for cyclic GMP. The chemical specificity required for increasing cyclic GMP was the same as that for stimulating calcium outflux, since for each agent studied, the dose response curve for stimulation of cellular cyclic GMP was the same as that for stimulation of calcium outflux. Furthermore, stimulation of neither calcium outflux nor cellular cyclic GMP depended in any way detectable on the presence of extracellular calcium and each could occur in a calcium-free, EGTA-containing medium. Finally, in cells incubated with CCK-OP or carbamylcholine the increase in cellular cyclic GMP was not associated with a decrease in cellular calcium per se, since EGTA or EDTA, which chelate extracellular calcium, abolish calcium influx, and decrease cellular calcium, did not increase cellular cyclic GMP. Our finding that for both calcium outflux and cellular cyclic GMP the increase produced by maximal concentrations of CCK-OP plus carbamylcholine was the same as that obtained with a maximal concentration of either agent alone suggests that both secretagogues stimulate the same effector mechanism. The ability of atropine to inhibit the effect of carbamylcholine but not that of CCK-OP indicates that these two agents interact with receptors which are functionally distinct.

Our findings might appear to differ from results obtained with segments of ductus deferens (24), slices of submaxillary gland (24), or segments of umbilical artery (25). With each of these tissues cholinergic and other agents increased cellular cyclic GMP when extracellular calcium was present. Preincubating and incubating the tissues in calcium-free solutions abolished cellular cyclic GMP while readdition of calcium restored the response. In contrast, in the present studies we found that CCK-OP, carbamylcholine, or A-23187 increased cellular cyclic GMP to the same extent in a calcium-free,
Results are expressed as the fraction of the amount of cyclic GMP solution also contained 5 mM EGTA. CCK-OP (10^{-7} M) stimulated cyclic GMP in dispersed pancreatic acinar cells. Cells were preincubated for 5 min at 37°C in standard incubation solution containing the corresponding calcium concentration but no CCK-OP. Varying extracellular calcium from 0 to 2.5 mM alone did not alter basal cyclic GMP. Values are means of three separate experiments ± 1 S.D.

EGTA-containing medium as in a calcium-containing medium. The apparent discrepancy may be related to differences in the extent to which the tissues can be depleted of calcium by the preincubation procedure. After preincubating dispersed pancreatic acinar cells for 40 min in a calcium-free, EDTA-containing medium, total exchangeable cell calcium is still approximately 30% of its initial value (7). We are not aware of data documenting the rate or extent of depletion of cellular calcium in ductus deferens, submaxillary gland, or umbilical artery incubated in a calcium-free solution. Our results with pancreatic acinar cells indicate that the increase in cyclic GMP produced by a particular agent is associated with mobilization and release of cellular calcium but not with increased inward movement of calcium. If a similar phenomenon occurs in ductus deferens, submaxillary gland, or umbilical artery the loss of stimulation following preincubation of the tissue in calcium-free medium could be due to loss of calcium from critical cellular sites during the preincubation, thereby preventing its subsequent mobilization by a particular stimulant.

Another possible explanation for the apparent discrepancy is that ductus deferens, submaxillary gland, and umbilical artery differ from pancreatic acinar cells in terms of extracellular calcium being required for stimulation of cyclic GMP in the former but not in the latter. Evidence for a variable role of calcium in mechanisms effecting cellular accumulation of cyclic GMP is the finding by Clyman et al. (25) that preincubating and incubating umbilical artery in a calcium-free solution abolishes the increase in cyclic GMP produced by histamine, acetylcholine, bradykinin, or potassium but not that produced by serotonin. Furthermore, Kimura and Murad (26) have found two apparently separable guanylate cyclases in rat heart—a soluble activity that is stimulated by calcium and a particulate enzyme that is inhibited by calcium.

In contrast to our finding that stimulation of cellular cyclic GMP is independent of external calcium, stimulation of pancreatic enzyme secretion by cholecystokinin or cholinergic agents is inhibited when calcium is removed from the incubation medium (27–32). The reported values for the extent of the inhibition have varied, and recently Williams and Chaudler (5) found that in mouse pancreas bethanechol increased release of amylase with or without extracellular calcium but that at all concentrations of bethanechol studied enzyme release was reduced by approximately 50% by removing extracellular calcium. In addition to the present studies, the divalent cation ionophore has also been observed to stimulate enzyme secretion from fragments of pancreas (22, 23) as well as potassium release from rat parotid gland (33). In each of these other studies ionophore-stimulated secretion was abolished when calcium was removed from the incubation medium. Some (34) have interpreted these observations as indicating the presence in the pancreas of a mechanism similar to "stimulus-secretion coupling" originally proposed by Douglas (35) to account for release of catecholamines from adrenal medulla. In this interpretation it is proposed that the secretagogue initiates influx of calcium, which then by some unknown sequence of events causes enzyme secretion. Some investigators have further extended this hypothesis by suggesting that receptors in the pancreas when activated by a stimulant function as ionophores to introduce calcium into the cell (34).

In contrast to the preceding hypothesis, cholecystokinin and muscarinic cholinergic agents have been demonstrated to increase outflux of calcium from intact pancreas (4, 6), pancreatic fragments (5), and dispersed pancreatic acinar cells (7). On the basis of these observations it has been proposed that these secretagogues increase pancreatic enzyme secretion by releasing intracellular "stored" calcium (4). This hypothesis also proposes that release of slowly exchanging cellular calcium is accompanied by an increase in free cytoplasmatic calcium, although no such increase has been demonstrated.

The divalent cation ionophore, A-23187, like CCK-OP and carbamylcholine, increased cellular 45Ca outflux and cyclic GMP and amylase secretion; however, unlike CCK-OP and carbamylcholine, the ionophore significantly increased the initial rate of 45Ca uptake. The ability of A-23187 to increase calcium uptake can be dissociated from its ability to increase calcium outflux and cyclic GMP, since the latter two phenomena occur in a calcium-free medium. This ability of the ionophore to increase cyclic GMP in a calcium-free medium indicates that the sequence of events under these conditions is increased calcium outflux, which in turn, increases cellular cyclic GMP. These findings also suggest that in pancreatic acinar cells the initial steps in the mechanism of action of CCK-OP and of carbamylcholine are mobilization of cellular calcium followed by increased cyclic GMP (4). This interpretation is based on the observation that the ionophore can promote transport of divalent cations across biological as well as artificial membranes (10) and the assumption that the ionophore cannot increase cellular cyclic GMP through a mechanism independent of enhanced calcium fluxes. * The observation that optimal stimulation of pancreatic enzyme

* Another observation which is consistent with, although certainly not compelling proof of this interpretation is that adding exogenous cyclic GMP or N6-O2′-dibutyryl cyclic GMP did not increase uptake or outflux of 45Ca in pancreatic acinar cells.
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secretion requires extracellular calcium indicates that this requirement is at a step distal to the initial events which characterize the action of pancreatic secretagogues and which do not require extracellular calcium. Furthermore, the studies by Williams and Chandler (5) indicate that extracellular calcium may be involved in the action of pancreatic secretagogues only in being required for optimal function of the secretory mechanism, but that extracellular calcium is not involved in the stimulation of the secretory mechanism per se.

REFERENCES


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Action of cholecystokinin, cholinergic agents, and A-23187 on accumulation of guanosine 3′:5′-monophosphate in dispersed guinea pig pancreatic acinar cells.
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