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EFFECTS OF AMINO ACIDS, GLUCAGON, INSULIN AND ACETYLCHOLINE ON CYCLIC NUCLEOTIDE METABOLISM AND AMYLASE SECRETION IN ISOLATED MOUSE PANCREATIC FRAGMENTS

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Abstract—The effects of amino acids, exogenous islet hormones and acetylcholine on cyclic nucleotide metabolism and amylase secretion in the isolated mouse pancreas have been investigated. The changes in levels of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) were measured at different times during exposure of pancreatic fragments to amino acids (L-alanine and L-arginine), islet hormones (insulin and glucagon) or acetylcholine (ACh). L-Alanine (1–20 mM) evoked a transient increase in cyclic AMP concentration accompanied by an initial decrease and subsequent increase in the tissue concentration of cyclic GMP. L-Arginine (1–20 mM) induced a complex triphasic change in cyclic AMP concentrations involving an initial rise and a delayed sustained elevation. The changes in levels of cyclic GMP increased only transiently. The effects of insulin (10^{-6} M) and to some extent glucagon (5×10^{-7} M) resembled those seen with L-arginine. The effects of amino acids and islet hormones were all dose-dependent. ACh (10^{-7} M) elicited a marked reduction in cyclic AMP concentration and this was accompanied by a concomitant increase in the level of cyclic GMP. The amino acids and the islet hormones had no significant effect on amylase secretion whereas ACh, of course, evoked a large increase in amylase output. The results with the amino acids and islet hormones reveal a clear dissociation between cyclic nucleotide changes and amylase secretion and further suggest that the marked reciprocal changes in cyclic AMP and cyclic GMP concentrations may constitute an important physiological role for the cyclic nucleotides to regulate amino acid transport in the pancreas.

The actions of secretin and vasoactive intestinal polypeptide (VIP) on pancreatic acinar cells are associated with changes in cyclic AMP levels [1, 2] whereas the actions of ACh, cholecystokinin-like and bombesin-like peptides are associated with release of intracellular Ca^{2+} and an increase in cyclic GMP concentration [1–3].

A number of amino acids can be taken up into cells by a sodium–amino acid co-transport mechanism [4–6]. Sodium gradient-driven amino acid transport across pancreatic plasma membranes has also been demonstrated [7] and a number of neutral amino acids like L-alanine and glycine, as well as the basic L-arginine, have been shown to depolarise and increase the conductance of the pancreatic acinar plasma membrane [8–11]. Amino acid transport mechanisms have been studied in the greatest detail in the intestine [12] and there are several reports which suggest a role for cyclic AMP in the regulation of amino acid transport into a variety of cells and organs [13–16], possibly mediated through changes in membrane sodium permeability [17–21].

The experiments described in this paper are concerned with the effects of amino acids, both the neutral L-alanine and the basic L-arginine, on cyclic AMP and cyclic GMP metabolism and amylase secretion in isolated mouse pancreatic fragments. There are several reports that arginine is a potent releaser of endogenous islet hormones [22, 23]. The action of exogenous insulin and glucagon on cyclic nucleotide changes and enzyme secretion was therefore also investigated mainly to test whether these hormones could mimic the actions of L-arginine.

MATERIALS AND METHODS

Perfusion procedure. All experiments were performed on small isolated segments (5–10 mg) of adult mice pancreas. Approximately 50–100 mg of tissues were placed into each of eight small Perspex flow chambers (volume = 1.0 ml). Four of the chambers served as 'test' preparations and the remaining four served as control 'partner' preparations. The tissues were superfused with Krebs Henseleit solution [composition (mM): NaCl, 103; KCl, 4.7; $CaCl_2$ 2.56; $MgCl_2$ 1.13; $NaHCO_3$ 25; NaH_2PO_4 1.15; D-glucose, 2.8; Na pyruvate 4.9; Na fumarate 2.7; and Na glutamate 4.9] at the rate of 1 ml/min. The solution was gassed with 95% O_2 and 5% CO_2 maintained at 37° and in all experiments (except those in which acetylcholine was used) atropine (10^{-5} M) was added. In the experiments in which the tissues were stimulated with either L-alanine or L-arginine, appropriate concentrations (range 1–20 mM) of either D-alanine or D-arginine were added to the control perfusing medium in order to maintain the same osmolarity throughout the experiment.

The tissue flow cells were perfused with Krebs solution for approximately 30–40 min prior to stimulation. During stimulation, the fluid flowing through the 'test' chamber was replaced with Krebs solution

containing appropriate concentrations of either L-alanine or L-arginine, insulin or glucagon or ACh. At various times during the onset of stimulation with the different agents, both the 'test' and control 'partner' preparations (i.e. tissue plus chamber) were rapidly frozen in liquid nitrogen and then stored until required for extraction and assay purposes.

Extraction and cyclic nucleotide assay procedures. Details of cyclic nucleotide extraction and assays are given elsewhere [24]. Frozen tissues were pulverised in a stainless-steel mortar and pestle (also cooled in liquid nitrogen) and afterwards extracted with acidic ethanol (1 ml of 1 N HCl in 100 ml of absolute alcohol). The solvent was blown off in a stream of gaseous nitrogen, and the residue taken up in Tris–EDTA buffer (0.05 M Tris, pH 7.5, containing 4 mM EDTA). Cyclic AMP and cyclic GMP levels were then determined using the Radiochemical Centre's assay kits, TRK 432 and TRK 500, respectively. Precise details of the procedures used are to be found in the publications which accompany the assay kits (Radiochemical Centre, Amersham, U.K.). Total protein was estimated using the Biuret method [25] and cyclic nucleotide concentrations obtained are expressed throughout in pmole/mg protein. The values plotted in the figures represent the difference in cyclic nucleotide concentrations between the 'test' and control 'partner' preparations.

Appropriate control experiments have shown that neither the amino acids nor the islet hormones nor ACh interfere with the cyclic nucleotide assays. The recoveries of cyclic AMP and cyclic GMP by the extraction procedure were approximately 90 and 91%, respectively.

Amylase secretion. Pancreata were cut into small segments (5–10 mg) and a total weight of around 100–150 mg placed in a Perspex flow chamber (volume = 1.0 ml) which was perfused with Krebs solution at a rate of 1 ml/min. Amylase in the effluent was assayed by the method of Rinderknecht and Marbach [26] as modified by Matthews *et al.* [27] and α-amylase (Sigma type IIA) was used as standard for calibration. The amino acids, islet hormones and secretagogues were added directly to the superfusing solution.

RESULTS

Mean control levels (±S.E.) of tissue cyclic AMP and cyclic GMP concentrations (pmole/mg protein) were: 2.94 ± 0.09 and 0.57 ± 0.03 (n = 131), respectively. There was some small fluctuation in control levels of cyclic nucleotides during the different experimental conditions, however, their levels remained virtually constant during a particular time course or dose-dependent response. A more detailed discussion of the variability in control levels of cyclic AMP and cyclic GMP is found elsewhere [24, 28]. The cyclic nucleotide concentration in a particular experimental situation was always expressed as the difference between the concentration in the test and the corresponding control preparation. Each data point in Figs. 1–5 therefore represents the difference between two single measurements. The significance of changes in cyclic nucleotide levels can perhaps be assessed by inspection of the dose-dependent response depicted in Figs. 1, 2 and 4.

Effects of amino acids on cyclic nucleotide levels

Figure 1 shows the time course of the changes in tissue cyclic AMP (A) and cyclic GMP (B) levels following superfusion of isolated pancreatic segments with different concentrations (range 1–20 mM) of L-alanine. Each point represents the cyclic nucleotide concentration (test minus control values) at a particular time following the onset of stimulation. L-Alanine evoked a rapid increase in cyclic AMP rising to a maximum within 30 sec of stimulation. This initial transient rise was followed by a slow decline reaching about 0.25 pmole/mg protein after 4 min. The early rapid increase in cyclic AMP was accompanied by a fall in cyclic GMP levels reaching a maximum after 30 sec. Thereafter, cyclic GMP rose, exceeding the control level (horizontal broken line) after 55–65 sec and continued to increase after 3–4 min. These time-dependent changes in endogenous cyclic AMP and cyclic GMP concentrations were dose-related (Fig. 1).

The L-arginine-evoked changes in cyclic nucleotide levels, expressed in pmole/mg protein and plotted as a function of time, are shown in Fig. 2 for different concentrations (range 1–20 mM) of the amino acid. L-Arginine induced a large and very rapid increase in endogenous cyclic AMP levels (A) reaching a maximum within 5–15 sec. This initial quick rise was followed by an abrupt fall to below the control level (horizontal broken line) after 20–40 sec. Cyclic AMP levels then rose again but more slowly to reach

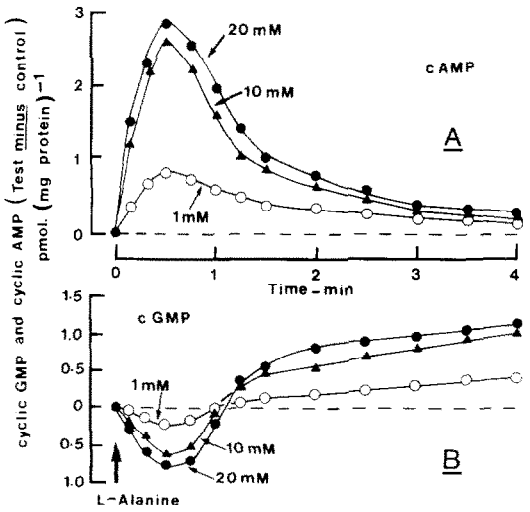

Fig. 1. Time course of changes in the tissue concentration of cyclic AMP (A) and cyclic GMP (B) during superfusion with different concentrations (range 1–20 mM) of L-alanine. Each point represents the difference in cyclic nucleotide concentration (pmole/mg protein) between two similar preparations, one serving as test and the other as a control. Atropine (10^{-5} M) was present throughout. The levels of cyclic AMP and cyclic GMP in control preparations were 2.48 ± 0.08 and 0.84 ± 0.04 pmole/mg protein, respectively (n = 39).

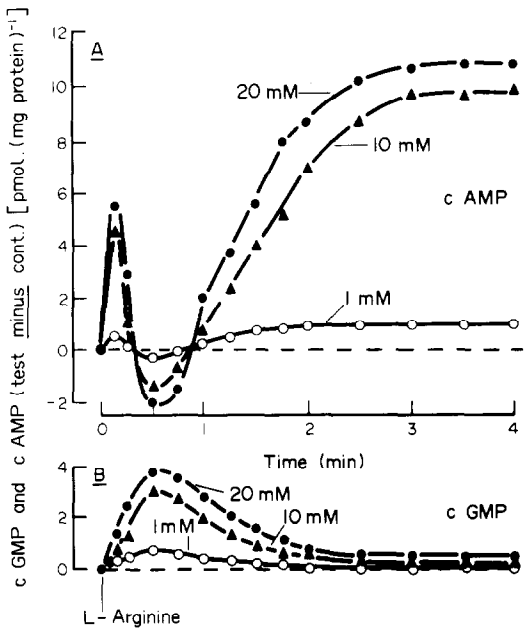

Fig. 2. Effects of L-arginine on time-dependent changes in endogenous cyclic AMP (A) and cyclic GMP (B) in mouse pancreatic segments. Each point represents cyclic nucleotide concentration (test minus control levels) at a precise time following onset of stimulation with varying concentrations (range 1–20 mM) of L-arginine. Atropine (10^{-5} M) was present throughout. Mean (±S.E.) control levels of cyclic AMP and cyclic GMP were 2.90 ± 0.09 and 0.47 ± 0.02 pmole/mg protein, respectively (n = 39).

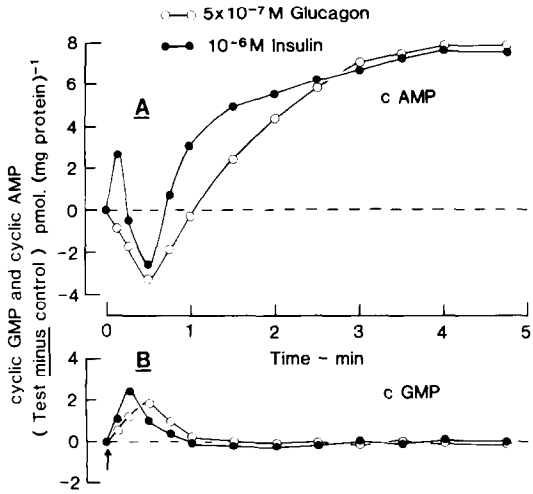

Fig. 3. Time course of changes in tissue cyclic AMP (A) and cyclic GMP (B) during superfusion of mouse pancreatic fragments with 5×10^{-7} M glucagon (open circles) and 10^{-6} M insulin (solid circles). Concentrations are expressed in pmole/mg protein and each point represents the test minus control values at a particular time following the onset of stimulation. Mean (±S.E.) control levels of cyclic AMP and cyclic GMP were 3.40 ± 0.15 and 0.42 ± 0.02 pmole/mg protein, respectively (n = 24).

a secondary maximum after 3–4 min. The early rapid rise and fall in cyclic AMP concentration was accompanied by a somewhat slower transient increase in cyclic GMP levels (B) reaching a maximum after 20–40 sec. It then declined slowly, returning to approximately the control level after about 2 min and stayed there throughout the remainder of the response. These time-course effects of L-arginine were dose-related (Fig. 2). In this series of experiments, a total of 150 preparations from 60 mice were used.

Effects of insulin and glucagon on cyclic nucleotide levels

There are several reports in the literature showing that arginine is a potent releaser of endogenous islet hormones [22, 23]. In this series of experiments the effects of exogenous insulin and glucagon on cyclic nucleotide levels were investigated in order to ascertain whether they could mimic the L-arginine-evoked changes in cyclic AMP and cyclic GMP concentrations.

Both insulin and glucagon were found to have potent stimulatory effects on the metabolism of cyclic AMP and cyclic GMP. Figure 3 shows changes in cyclic AMP (A) and cyclic GMP (B) concentrations (expressed as test minus control values) measured at different times during responses produced by 10^{-6} M insulin (solid circles) and 5×10^{-7} M glucagon (open circles). Insulin induced a rapid increase in cyclic AMP levels rising to about 2.5 pmole/mg protein within 5–10 sec. This initial rise was followed by an abrupt fall, to around −2.65 pmole/mg protein after 25–35 sec. Cyclic AMP levels then rose again to reach a secondary increase of around 7.0–7.5 pmole/mg protein after 3–4 min. The early rise and fall in cyclic AMP are accompanied by a somewhat transient increase in cyclic GMP concentrations, reaching a value of about 2.4 pmole/mg protein after 12–20 sec, cyclic GMP then declined slowly, returning to the control value after 50–60 sec and stayed there throughout the remainder of the response.

In the presence of 5×10^{-7} M glucagon, the changes in cyclic AMP levels fell during the first 15–45 sec to around −3.20 pmole/mg protein. Thereafter, cyclic AMP rose exceeding the control level (horizontal broken line) after 60–90 sec, reaching a maximum of around 7.5 pmole/mg protein after 3.5–4.5 min. The initial decrease in cyclic AMP levels is seen to coincide with a transient rise in intracellular cyclic GMP. The change in the level of cyclic GMP increased to around 1.85 pmole/mg protein after 30–40 sec and then continued to decline reaching the control value after 80–90 sec and remained there throughout the response. In this series of experiments a total of 48 preparations from 24 mice were tested.

Dose-related effects of insulin and glucagon on cyclic nucleotide levels

The ability of insulin and glucagon to stimulate cyclic AMP metabolism is dose-related whereas both substances produced little or no effect on cyclic GMP levels. Log dose–response curves showing the

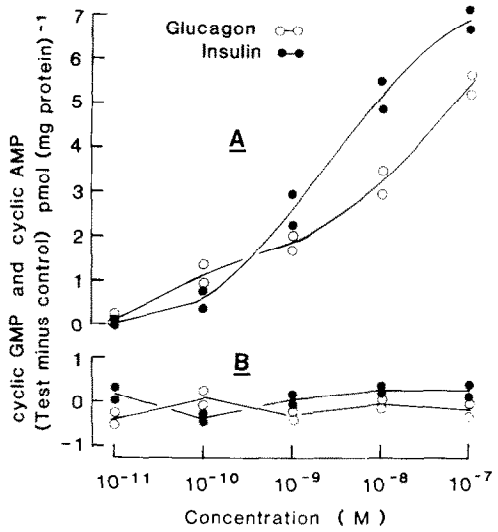

Fig. 4. Dose–response curves showing the effects of insulin (10^{-11}–10^{-7} M; solid circles) and glucagon (10^{-11}–10^{-7} M; open circles) on cyclic AMP (A) and cyclic GMP (B) levels. All values expressed as test minus control levels and preparations were perfused for 4 min prior to freezing. Atropine (10^{-5} M) was present throughout. The levels of cyclic AMP and cyclic GMP in control preparations were 1.91 ± 0.13 and 0.55 ± 0.03 pmole/mg protein, respectively (n = 20).

effects of different concentrations (10^{-11}–10^{-7} M) of insulin (closed circles) and glucagon (open circles) on changes (test minus control values) in cyclic AMP (A) and cyclic GMP (B) are presented in Fig. 4. All measurements were made on tissues which were frozen 4 min after commencing treatment with either insulin or glucagon. The data show that the pancreas is very sensitive to both islet hormones. Concentrations of 10^{-10} M produced a detectable effect on the metabolism of cyclic AMP whereas 10^{-11} M (the lowest concentration tested) produced little or no increase. In this series of experiments a total of 30 preparations from 10 mice were tested.

Time course of the changes in cyclic nucleotides during the response to acetylcholine

The effects of ACh (10^{-7} M) on the tissue concentration of cyclic AMP and cyclic GMP were investigated in a number of experiments. The changes in cyclic nucleotide levels, expressed as test minus control values and plotted as a function of time, are shown in Fig. 5A. Acetylcholine induced a relatively slow decline in cyclic AMP levels (open circles) reaching a steady-state level (around −4.0 pmole/mg protein after 2–3 min. This low level stayed throughout the remainder of the response. The decrease in cyclic AMP level was accompanied by a somewhat gradual increase in cyclic GMP level

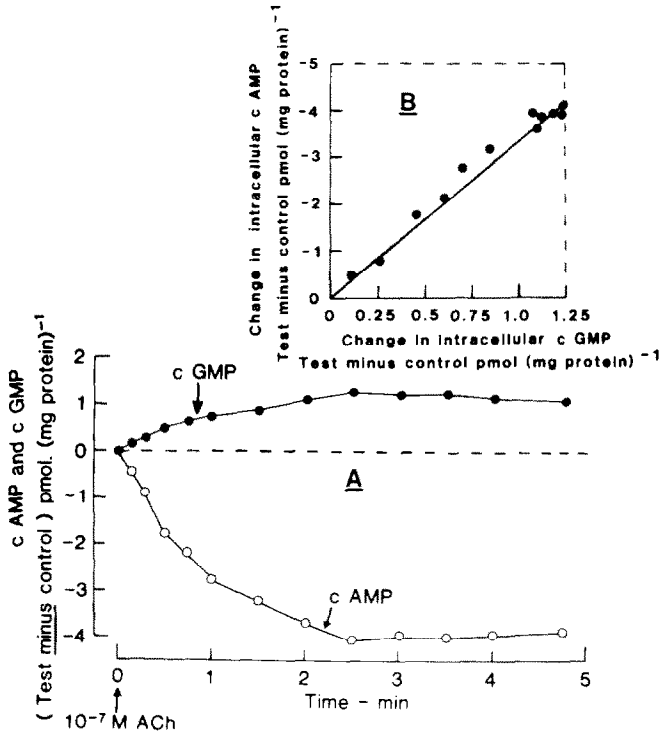

Fig. 5. (A) Time course of changes in cyclic AMP (open circles) and cyclic GMP (solid circles) during superfusion with 10^{-7} M acetylcholine (ACh). Mean (±S.E.) control levels of cyclic AMP and cyclic GMP were 4.08 ± 0.21 and 0.33 ± 0.02 pmole/mg protein, respectively (n = 12). (B) Inverse relationship between changes in endogenous cyclic AMP and cyclic GMP levels (test minus control levels) following treatment of pancreatic fragments with 10^{-7} M acetylcholine (data taken from Fig. 5A). The slope of the line is fitted by linear regression analysis.

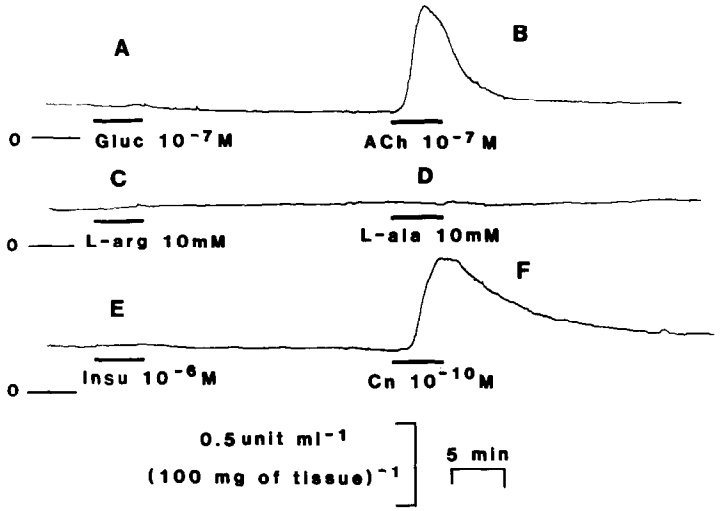

Fig. 6. Effects of 10^{-7}M glucagon (A), 10^{-7}M ACh, (B), 10 mM L-arginine (C), 10 mM L-alanine (D), 10^{-6}M insulin (E) and 10^{-10}M caerulein (F) on amylase release from pancreatic fragments. The responses A–F are continuous recordings of fluorescence intensity from a single experiment. The horizontal bars indicate the duration of stimulation. Vertical calibration 0.5 unit/ml per 100 mg tissue. The horizontal lines labelled ○ represent the fluorescence reading without pancreatic tissue in the flow cell.

(solid circles) reaching about 1.1 pmole/mg protein after 2–3 min, and remaining at that level throughout the response.

The reciprocal relationship between changes in cyclic AMP and cyclic GMP is shown graphically in Fig. 5B. Here the decrease in endogenous cyclic AMP concentration is plotted as a function of the increase in intracellular cyclic GMP. The relationship is approximately linear, yielding a slope of around -3.30 ± 0.11 ($n = 12$; $P < 0.001$) pmole cyclic AMP/pmole increment in cyclic GMP. This observation may suggest a possible role for cyclic GMP in suppressing endogenous cyclic AMP levels. In this series of experiments a total of 24 preparations from 12 mice were used.

Effects of ACh, amino acids and islet hormones on amylase secretion

Pancreatic amylase output was measured using the automated amylase assay system [27]. After the basal amylase output had attained a steady-state level the preparation was subsequently exposed to Krebs solution containing either the secretagogues, or amino acids or islet hormones. Figure 6 shows original chart recordings of responses produced by superfusing a preparation with 10^{-7}M glucagon (A), 10^{-7}M ACh (B), 10 mM L-arginine (C), 10 mM L-alanine (D), 10^{-6}M insulin (E) and 10^{-10}M caerulein (F). Both ACh and caerulein increased amylase secretion markedly above basal levels whereas the amino acids and islet hormones had no statistically significant effect (see Table 1 for data). Exposure of pancreatic fragments to 10^{-7}M ACh resulted in a mean peak secretion of 3.99 ± 0.26 unit/ml per 100 mg tissue. In the presence of 10^{-10}M caerulein, amylase output increased to 3.20 ± 0.27 unit/ml per 100 mg tissue.

DISCUSSION

The results of the experiments described here show that the amino acids and islet hormones

Table 1. Effects of ACh, insulin, glucagon, L-arginine, L-alanine and caerulein on amylase secretion

Experimental condition	Amylase secretion Peak response (unit/ml per 100 mg tissue ± S.E.)	N	P values
Control	1.04 ± 0.07	12	
ACh (10^{-7}M)	3.99 ± 0.26	12	$p < 0.001$
Control	1.04 ± 0.05	11	
Insulin (10^{-6}M)	1.03 ± 0.04	11	$p < 0.85$
Control	1.03 ± 0.05	16	
Glucagon (10^{-7}M)	1.13 ± 0.09	16	$p < 0.35$
Control	0.98 ± 0.05	11	
L-Arginine (10 mM)	1.13 ± 0.07	11	$p < 0.15$
Control	1.03 ± 0.08	9	
L-Alanine (10 mM)	0.97 ± 0.07	9	$p < 0.85$
Control	0.93 ± 0.06	8	
Caerulein (10^{-10}M)	3.20 ± 0.27	8	$p < 0.001$

markedly stimulate the production of both cyclic nucleotides and that this is associated with no significant change in amylase secretion. On the other hand, the effect of ACh is characterised by a marked reduction in the concentration of cyclic AMP and a concomitant increase in the levels of cyclic GMP. These changes are, of course, associated with an increase in pancreatic amylase output.

There are several reports demonstrating that the actions of some secretagogues on amylase secretion are associated with changes in cyclic nucleotide levels. Both VIP and secretin stimulate amylase secretion and this response is thought to be mediated via an increase in cellular cyclic AMP concentration [1, 2]. Our results with ACh are consistent with the previous findings of others regarding cyclic GMP, but also reveal for the first time that ACh induces a marked reduction in cyclic AMP concentration in the mouse pancreas. We have previously observed that caerulein decreases the concentration of cyclic AMP in the guinea-pig pancreas [24]. It was earlier suggested by Albano *et al.* [29] that pancreatic enzyme secretion in response to ACh may be triggered by a transient elevation in the intracellular cyclic GMP:cyclic AMP ratio. Interestingly, our results with ACh also show a large increase in the cyclic GMP:cyclic AMP ratio.

Stimulation with both the neutral L-alanine and the basic L-arginine is associated with qualitatively and quantitatively different time-dependent changes in pancreatic cyclic nucleotide concentrations. The dose–response curves for these changes are similar to the dose–response curves for acinar cell membrane depolarization [8–11]. L-Arginine clearly evokes much more pronounced changes in both cyclic AMP and cyclic GMP levels than L-alanine, while it is known that it exerts much less of a depolarizing effect on the acinar cells. The present results also reveal that both glucagon and insulin have marked stimulatory effects on pancreatic cyclic nucleotide metabolism. However, there is no direct evidence to suggest whether the changes in cyclic nucleotide levels are occurring mainly in those cells which secrete amylase. It is worth mentioning, in passing, that insulin receptors have been demonstrated in pancreatic acinar cells and it has been suggested that insulin may directly regulate specific functions in the exocrine pancreas [30]. Interestingly, the nature of the time-course changes in cyclic nucleotide levels seen with insulin and to some extent glucagon resembles the changes obtained with L-arginine. These observations may suggest some indirect action of L-arginine on cyclic AMP and cyclic GMP metabolism in the pancreas, especially since L-arginine is a potent releaser of endogenous islet hormones [22, 23].

The results of this investigation also show that both the amino acids and islet hormones have no significant effect on pancreatic amylase secretion. However, there are several other conflicting reports which demonstrated variable actions of glucagon and insulin on the exocrine pancreas. Glucagon has been shown to inhibit the secretion of amylase *in vivo* [31, 32]. In isolated mouse pancreas one report suggests no change [33] whereas another demonstrated a large increase in amylase output in response to an unphysiological dose (10^{-4} M) of glucagon [34]. On the other hand, insulin alone has no effect on amylase secretion, but it can markedly potentiate the secretory responses of CCK and acetylcholine [35, 36]. The changes in cyclic AMP levels obtained following stimulation with insulin in this study may in fact be associated with its potentiating action. Some recent experiments (unpublished data) in our laboratory have demonstrated that insulin has no effect on the ACh-evoked $^{45}Ca^{2+}$ efflux whereas it markedly enhanced cyclic AMP concentration in the presence of the cholinergic agonist. Nevertheless, the present data with the amino acids and islet hormones reveal a clear dissociation between changes in cyclic nucleotide metabolism and amylase output in the mouse pancreas. Recently, several workers have reported a dissociation between amylase secretion and elevated cyclic nucleotide levels during stimulation of the pancreas with nitrosourea compounds [37, 38] and secretin [39, 40]. Experiments with CO_2 application reveal a marked release of intracellular Ca^{2+} which is associated with a reduction in amylase output [41]. Dissociation between cyclic nucleotide changes and physiological parameters (e.g. contraction) has also been observed in the heart [42, 43].

There are several indications of possible links between amino acid transport across cell membranes and cyclic nucleotide metabolism. More recently, cyclic AMP has been found to enhance significantly the uptake of amino acids (both neutral and basic) in the presence of sodium into a variety of tissue types including the kidney cortex [17], liver [16, 21, 44], bone [18, 19], brush border membrane vessicles [20] and the intestine [12–15]. It has been suggested that cyclic AMP activates a sodium-sensitive protein kinase which in turn phosphorylates a membrane-bound regulatory protein thereby enhancing the uptake of the amino acids [45]. The present results clearly show that both L-alanine and L-arginine markedly stimulate the production of cyclic AMP and cyclic GMP in the mouse pancreas. The most straightforward interpretation of our data is to postulate that the initial sequence of events in L-alanine and L-arginine transport pathways is activation of cyclic nucleotide metabolism. Cyclic AMP in turn stimulates the sodium-sensitive protein kinase which then phosphorylates the membrane-bound regulatory (carrier) protein thereby enhancing the translocation events. On the other hand, cyclic GMP may have the opposing effect (dephosphorylation of the membrane protein) resulting in a change in substrate transport rate.

Our study of time-dependent and dose-related cyclic nucleotide concentration changes following stimulation with a variety of substances has revealed an interesting reciprocal pattern of changes in cyclic AMP and cyclic GMP. Similar reciprocal patterns have also been described in the guinea-pig pancreas in response to stimulation with secretagogue peptides [24], in the heart [46] and the thyroid gland [28]. This is illustrated by three specific examples presented in this study. Firstly, the action of L-alanine is characterised by an increase in cyclic AMP level which is preceded by a transient fall in the level of cyclic GMP. The opposite relationship is seen

later during the same response. Secondly, the cyclic nucleotide changes evoked by L-arginine, insulin and glucagon display a reciprocal pattern in the sense that the peak in cyclic GMP concentration is reached at the time of minimum cyclic AMP concentration while the subsequent minimum cyclic GMP concentration was reached at the time of the second peak of cyclic AMP concentration. Thirdly, the response to ACh is characterised by an increase in cyclic GMP concentration and this is linearly related to the accompanying fall in cyclic AMP concentration. These three observations suggest the possibility that one cyclic nucleotide may constitute part of a feed-back control mechanism which serves to regulate the metabolism of the other. This is an interesting area which deserves further investigation.

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