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HORMONAL STIMULATION OF cAMP-DEPENDENT PROTEIN KINASE IN RAT PANCREAS

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SUMMARY

Rat pancreas possesses a cAMP-dependent protein kinase enzyme which is inhibited by the protein kinase inhibitor from bovine heart. Incubation of rat pancreas tissue in the presence of secretin and CCK-PZ resulted in an increase of intracellular cAMP and activation of the cAMP-dependent protein kinase. CCK-OP, a pure synthetic analog of CCK-PZ, did not stimulate cellular cAMP production or protein kinase activation. There was correlation between the ability of a given dose of hormone to stimulate cAMP formation and activate the protein kinase enzyme. It is believed that the stimulatory effect of CCK-PZ is due to secretin contamination of this hormonal preparation.

Many of the effects of cyclic adenosine monophosphate (cAMP)in mammalian tissues are believed to be mediated by changes in the activity of cAMP-dependent protein kinase (1). Cyclic AMP activates protein kinase by combining with the regulatory subunit of the enzyme and liberating an active catalytic subunit. The catalytic subunit phosphorylates the appropriate substrate, resulting in an intracellular expression of hormonal action (2). Soderling and associates (3) showed that hormonal activation of protein kinase by cAMP can be studied in vitro using tissue slices.

cAMP has been implicated as the second messenger of hormonally stimulated exocrine pancreas. Secretin and vasoactive intestinal peptide increased cAMP levels in acinar cells isolated from guinea pigs (4,5). Cholinergic agents and cholecystokinin (CCK) had no effect on cAMP levels, but caused an increase in tissue levels of cyclic guanosine monophosphate (cGMP). However CCK has been shown to stimulate adenylyl cyclase in the pancreas of rat and guinea pig (6,8).

The aim of the present work was to study the activation of cAMP-dependent protein kinase by stimulation of endogenous cAMP formation in the presence

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of hormones. It was of special interest to compare the effects of secretin with cholecystokinin-pancreozymin (CCK-PZ), and a synthetic octapeptide of cholecystokinin (CCK-OP).

MATERIALS AND METHODS

Tissue Incubation. Pancreata were obtained from male Sprague-Dawley rats weighing 150 to 200 gm fed on a standard meal ad libitum. Prior to the experiment, the rats were fasted for 48 hours. The rats were killed by a blow to the head; the pancreas quickly removed, dissected free from fat and mesentery and used immediately. The pancreas was cut into small pieces and placed in Ringer's bicarbonate buffer, pH 7.4, containing 1 mg/ml glucose and 0.5 mM 3-isobutyl-1-methyl xanthine (IMX). The tissue was preincubated at 37° C for ten minutes with mild shaking. At the end of the preincubation period, the tissue was transferred to fresh buffer containing the appropriate hormones and incubated for additional ten minutes at 37° C. Two to three pieces of tissue, approximately 2 to 4 mm^2, were incubated in 5 ml of buffer. At the end of the incubation period, the tissue was quickly removed, frozen in liquid nitrogen, and subsequently stored under liquid nitrogen.

Enzyme Preparation. Frozen tissue was pulverized in liquid nitrogen and the pulverized powder homogenized in ten volumes of 10 mM potassium phosphate buffer, pH 6.5, containing 10 mM ethylene diamine tetraacetate (EDTA) and 0.5 mM IMX. The homogenate was centrifuged at 27,000 x g for 20 minutes at 4° C. The supernatant fraction was used as the enzyme preparation. Protein kinase assay was performed immediately after enzyme preparation.

Protein Kinase Assay. The reaction was started by adding 5 to 20 µl of the enzyme preparation to 50 µl of a solution containing 17 mM potassium phosphate buffer, pH 6.8, 10 mg/ml histone (Sigma type II-A), 6 mM magnesium acetate, 0.33 mM ATP-γ^{32}p (20 to 50 counts per minute/pmole) in the presence and in the absence of 2 µM cAMP. The reaction mixture was incubated at 30° C for 5 minutes and the reaction terminated by addition of 1 ml of ice cold 10% trichloroacetic acid (TCA). The formed precipitate was collected by filtration on Whatman GF/C glass fiber filters, washed four times with 2 ml portions of ice cold 10% TCA, and counted in 5 ml of Bray's solution (9) in a Packard Tri-Carb liquid scintillation counter. The protein kinase activity was expressed as pmoles of ^{32}p incorporated into histone per milligram of protein per minute. The protein kinase activity ratio was expressed as the ratio of protein kinase activity in the absence of added cAMP to protein kinase activity in the presence of 2 µM cAMP. In each assay nonspecific binding of ^{32}p to histone was determined by measuring the amount of ^{32}p bound to denatured enzyme. The value obtained was used as zero time incubation, and was subtracted from all other values.

cAMP Assay. Frozen tissue, previously incubated with and without hormones, was homogenized in 3 ml of ice cold 6% TCA and 6000 dpm of ^{3}H-cAMP were added to determine recovery. The homogenate was centrifuged at 10,000 x g for 30 minutes at 4° C. The supernatant fraction was applied to a Dowex 50-H^+ form column, 100 to 200 mesh, 0.9 x 15 cm, which had been equilibrated with 0.1N HCl. The column was eluted with 0.1N HCl, the cAMP elution profile determined, and the fraction containing cAMP was collected and lyophilized to dryness. The lyophilized fraction was resuspended in 1 ml of water and the concentration of cAMP was determined against a standard curve by the method of Gilman (10). The cAMP values obtained were corrected for recovery and expressed as pmoles of cAMP per milligram of protein.

Protein Determination. Protein concentration was determined by the method of Lowry (11).

Materials. Type II-A histone, cAMP-dependent protein kinase inhibitor from bovine heart, cAMP, ATP, IMX, and binding protein were purchased from the Sigma Chemical Co. The natural porcine preparation of secretin and CCK-PZ were purchased from the Karolinska Institute, Stockholm, Sweden; this supplier states that each ampule containing 75 U of CCK-PZ also contains 0.7 U of secretin. CCK-OP was a gift of the Squibb Institute for Medical Research; ATP-γ^{32}P and cAMP-^{3}H were purchased from the New England Nuclear Corporation. All other chemicals used were of the highest purity commercially available.

RESULTS

Control Studies. Addition of exogenous cAMP to the reaction mixture caused a concentration-dependent increase in pmoles of ^{32}P incorporated into histone (Fig. 1). Activation of protein kinase was already apparent at a cAMP concentration of 0.05 µM and was optimal at 0.4 µM, when the concentration of the enzyme was 200 µg protein.

The effects of protein concentration and time of incubation on protein kinase activity are shown in Fig. 2. In all subsequent experiments the reaction was carried out for five minutes at 30° C maintaining protein concentration between 100 to 500 µg per tube.

It has been shown (12,13) that in some tissues addition of NaCl has a stabilizing effect on the protein kinase activity ratio. This effect is probably caused by stabilization of dissociation of the protein kinase holoenzyme. Increasing concentrations of NaCl present during tissue homo-

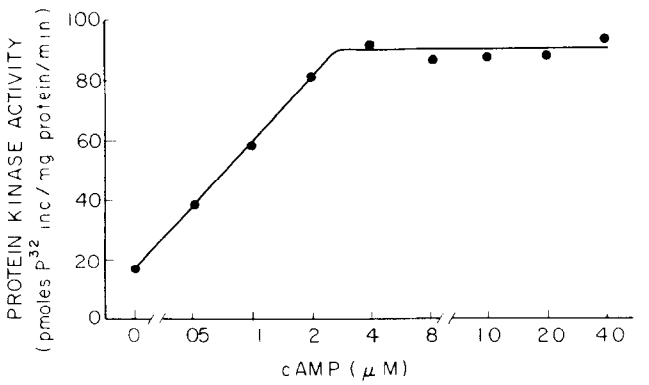

Figure 1. Effect of cAMP on protein kinase activity. The enzyme (200 µg protein) was incubated for 5 minutes at 30° C in the presence of varying cAMP concentrations, and protein kinase activity was determined. Each point was determined in triplicate, and this experiment is representative of two others.

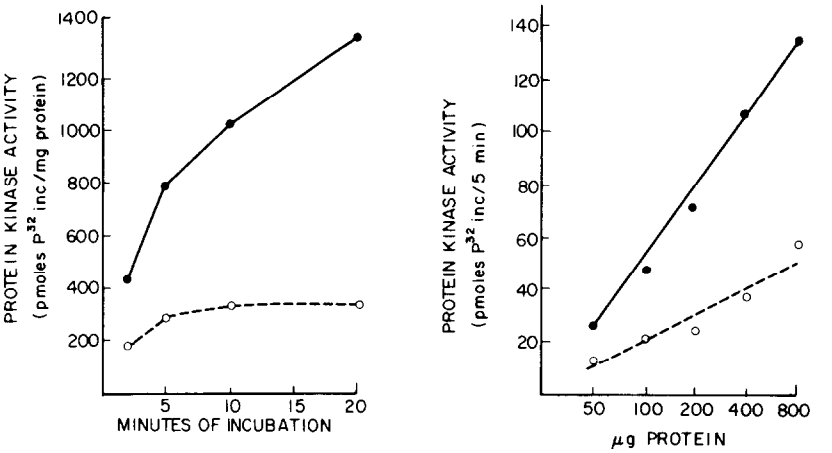

Figure 2. Effects of incubation time and protein concentration on the protein kinase activity. In the top curve 100 µg protein were incubated for different times at 30° C in the presence and absence of 2 µM cAMP. In the bottom curve varying amounts of protein were incubated for 5 minutes at 30° C in the presence and absence of 2 µM cAMP. Each point was determined in triplicate and each experiment is representative of two others. The dashed line represents values in the absence of cAMP; solid line represents values in the presence of 2 µM cAMP.

genization caused an increase in protein kinase activity ratio (Fig.3), indicating an increase in the catalytic component of the enzyme probably due to dissociation of the protein kinase holoenzyme. NaCl was therefore omitted in the following experiments.

The cAMP-stimulated portion of the protein kinase activity was inhibited by a cAMP-dependent protein kinase inhibitor from bovine heart (Fig. 4). At 80 µg of inhibitor protein, protein kinase activity approached that in the absence of cAMP.

Hormonal Effects. As shown in Fig. 5, cAMP levels in the tissue rose after incubation with secretin or CCK-PZ. The increase was already apparent at a secretin concentration of 10^{-7} M. When secretin concentration was 10^{-5} M, tissue levels of cAMP were approximately nine times the basal level. There was no further increase in cAMP level when secretin concentration was raised to 10^{-4} M. The natural porcine preparation of CCK-PZ also caused an increase in tissue cAMP level but higher concentrations of CCK-PZ were required to produce a response of the same order of magnitude as obtained with secretin.

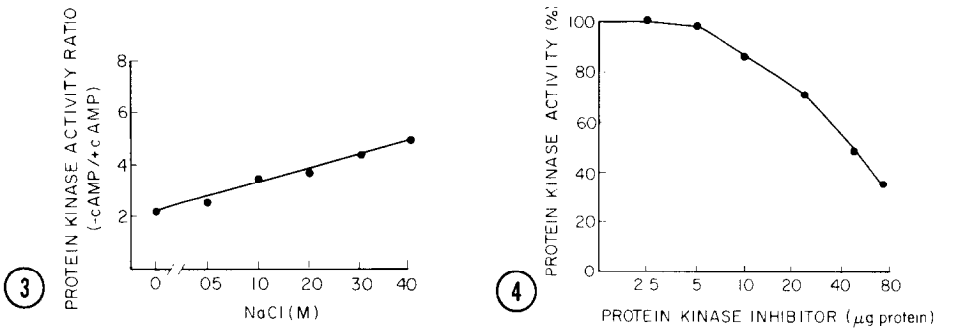

Figure 3. Effect of NaCl on protein kinase activity ratio. Varying concentrations of NaCl were present during homogenization of the tissue. The enzyme was incubated in the presence and absence of 2 μM cAMP for 5 minutes at 30° C. Each point was determined in triplicate, and this experiment is representative of two others.

Figure 4. Effect of bovine heart protein kinase inhibitor on protein kinase activity. The enzyme (47 μg protein) was incubated with varying concentrations of the protein kinase inhibitor in the presence of 2 μM cAMP for 5 minutes at 30° C. The protein kinase activity in the absence of 2 μM cAMP was the same as that in the presence of 75 μg protein of the protein kinase inhibitor. Protein kinase activity is expressed as per cent of the activity obtained with 2 μM cAMP in the absence of protein kinase inhibitor.

The synthetic carboxy-terminal octapeptide (CCK-OP) did not produce significant changes in cAMP tissue levels. At 10^{-4} M CCK-OP, tissue cAMP levels were the same as controls.

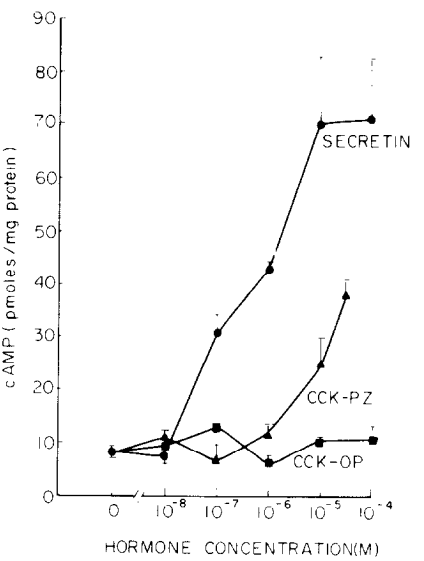

Figure 5. Effect of hormone concentration on cAMP levels. The tissue was preincubated with buffer alone for 10 minutes and then incubated with hormones for additional 10 minutes. Each point is a mean ± s.e. of three separate experiments, and each experimental value was obtained from quadruplicate tissue samples.

Table I EFFECT OF HORMONES ON PROTEIN KINASE ACTIVITY RATIO

	Protein Kinase Activity Ratio (-cAMP/+cAMP)		
Hormone Concentration (M)	Secretin	CCK-PZ	CCK-OP
0	0.188 ± .006	0.188 ± .006	0.245 ± .017
10^{-8}	0.220 ± .010	0.198 ± .033	0.274 ± .019
10^{-7}	0.271 ± .000	0.233 ± .012	0.252 ± .009
10^{-6}	0.315 ± .016	0.245 ± .022	0.288 ± .029
10^{-5}	0.419 ± .010	0.300 ± .036	0.293 ± .025
10^{-4}	0.482 ± .000	0.355 ± .036	0.273 ± .029

Tissue was preincubated with buffer alone for 10 minutes, and then incubated with hormones for additional 10 minutes. Protein kinase activity ratio was determined as described under methods. Each point is a mean ± s.e. of three separate experiments, and each experimental value was obtained from quadruplicate tissue samples.

Incubation of pancreatic tissue with secretin or CCK-PZ produced a concentration-dependent increase in the protein kinase activity ratio (Table I). The ratio increased from 0.188 ± 0.066 without any added hormone to 0.482 ± .000 in the presence of 10^{-4} M secretin. Incubation of tissue with 10^{-4} M CCK-PZ produced an increase in the protein kinase activity ratio to 0.355 ± .036. The synthetic CCK-OP did not produce any significant change in protein kinase activity ratio when tested between 10^{-8} to 10^{-4} M. The higher basal activity ratio in the CCK-OP experiments cannot be explained but may have been caused by animal and experimental variations.

DISCUSSION

Stimulation of exocrine pancreatic secretion is thought to be related to changes in tissue levels of cAMP (14). It has been shown that stimulation of fluid and bicarbonate secretion is mediated through the cAMP-adenylyl cyclase system (15-20). On the other hand, although data point to the noninvolvement of cAMP during CCK-stimulated enzyme secretion (4),some reports have shown stimulation of adenylyl cyclase activity and increased cellular cAMP after administration of the hormone (8,21-24). The metabolic and functional effects of increased cAMP levels are believed to be expressed through the activation

of cAMP-dependent protein kinases. The presence of a cAMP-dependent protein kinase has been demonstrated in homogenates of pancreata from rats (25-27) and in acinar cells isolated from the pancreata of guinea pigs (28). Our study has confirmed that the supernatant fraction of the homogenate of rat pancreas contains a protein kinase enzyme which is stimulated by cAMP. In addition, this study demonstrates hormonal stimulation of the cAMP-dependent protein kinase using an in vitro system.

Incubation of pancreatic tissue in Ringer's bicarbonate buffer with secretin or CCK-PZ produced a dose-dependent increase in tissue levels of cAMP. Secretin was approximately 100-fold as potent a stimulant as was CCK-PZ. In concentration ranges between 10^{-8} to 10^{-4} M CCK-OP, the synthetic analog of CCK-PZ, did not elevate tissue cAMP levels.

There was a general relationship between the degree of potency of the hormones in elevating cAMP tissue levels and activating the protein kinase enzyme. When it is assumed that the stimulation of the cAMP-dependent protein kinase activity can be achieved only by stimulation of endogenous production of cAMP, the present findings strengthen the current concept that secretin activates an adenylyl cyclase enzyme in the pancreas, resulting in increased production of intracellular cAMP. This newly formed cAMP activates a protein kinase with subsequent stimulation of fluid secretion. The results with the two different preparations of cholecystokinin (CCK-PZ and CCK-OP) indicate that the hormonal moiety which is responsible for stimulation of pancreatic enzyme secretion is not responsible for the stimulation of cAMP and protein kinase activation, since CCK-PZ and CCK-OP are equipotent stimulants of enzyme secretion. It is known that the CCK-PZ preparation is contaminated by secretin. Taking into account the amount of secretin in the CCK-PZ preparation (see Materials) and the molecular weights of the two hormones, it can be calculated that each mMole of the CCK-PZ preparation contains approximately 2×10^{-3} mMoles of secretin. This secretin can account for the degree of stimulation found with the CCK-PZ preparation.

Complete activation of the protein kinase enzyme was not achieved. It is possible that a rapid reassociation of the catalytic and regulatory subunits of the protein kinase enzyme occurs during tissue homogenization and assay, resulting in a lower activity ratio. Preliminary experiments indicate that presence of 0.5 M NaCl during tissue homogenization results in a higher protein kinase activity ratio both in the absence and presence of hormones. In summary, the present findings confirm that cAMP is not involved during hormonally stimulated pancreatic enzyme secretion, and stimulation of adenylyl cyclase by cholecystokinin may be attributed to secretin contamination of this hormonal preparation.

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