CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES OF THE RAT PANCREAS.

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SUMMARY. A cyclic GMP-dependent protein kinase, which catalyzes the phosphorylation of histones and protamine by ATP, was present together with a cyclic AMP-dependent protein kinase and a readily active protein kinase in the rat pancreas. These three protein kinases were separated by chromatography on DEAE-cellulose. The cyclic GMP-dependent protein kinase was relatively cationic and fragile. Upon activation by cyclic GMP, this kinase dissociated into a light catalytic subunit and a somewhat heavier cyclic GMP binding subunit. A crude 27,000 x g pancreas supernatant had two apparent K_a values for cyclic GMP of 2.10^{-8} M and 3.10^{-7} M. The possible relationships between protein kinases and enzyme secretion are discussed.

It has been demonstrated recently that the proteins of rat pancreas are more actively phosphorylated in vitro in the presence of pancreozymin and caerulein (1). This increased rate of phosphorylation is especially important in the membrane of zymogen granules, i.e. in proteins directly involved in "stimulus-secretion coupling". Carbamylcholine also stimulates protein phosphorylation under similar incubation conditions (unpublished data). These observations suggest the participation of protein kinase(s). To our knowledge, there has been only one report (2) indicating cyclic AMP-dependent protein kinase activity of average intensity in the pancreas. An account of our results was presented at the 6th Symposium of the European Pancreatic Club (Göteborg, May 24-26, 1973).

MATERIALS AND GENERAL METHODS.

Rat pancreases were obtained from 150-200 g animals of both sexes fed ad libitum on a standard chow. Cyclic nucleotides were purchased from Boehringer (Mannheim, Germany). [γ-^{32}P] ATP was obtained from the Radiochemical Centre (Amersham, England). ATP, protamine sulfate (grade II) from salmon and histones (Type II A) from calf thymus were purchased from Sigma (St. Louis, U.S.A.).

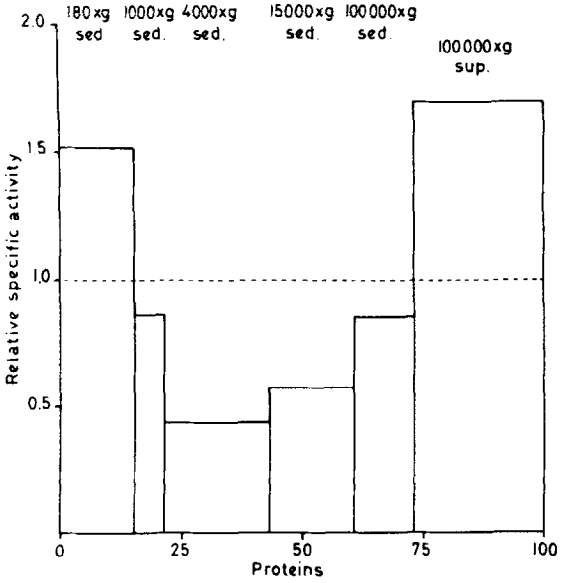

FIGURE 1. SUBCELLULAR DISTRIBUTION OF BASAL PROTEIN KINASE ACTIVITY.
The 6 crude fractions from rat pancreas separated by differential centrifugation in 10 mM Tris-maleate buffer (pH 6.0) - 0.3 M sucrose (as described in ref. 1) were nuclear (180 x g x 10 min sed), zymogen granule (1000 x g x 10 min sed), mitochondrial (4000 x g x 10 min sed), heavy microsomal (15,000 x g x 15 min sed), light microsomal (100,000 x g x 60 min sed), and supernatant (100,000 x g x 60 min sup). Relative activities are the ratios of the activity in the fraction under consideration to the average activity in complete homogenate. The width of each bar represents the percentage of total proteins recovered in each fraction. Values are the average of 4 experiments.
The basal activity of protein kinase(s) was assayed under conditions of endogenous substrate availability in a final volume of 0.2 ml containing 10 µmoles of sodium phosphate buffer (pH 6.5); 40 nanomoles of [γ-^{32}P]ATP (1 x 10^6 cpm), 2 µmoles of magnesium sulfate, 2 µmoles of sodium fluoride, 0.2 µmoles of aminophylline, and 1 µmole of EDTA. The mixture was incubated at 30° for 5 min and the protein-bound ^{32}P was determined as described by KUO and GREENGARD (4).

Protein was measured by the method of Lowry et al. (3). Protein kinase activity in pancreas homogenates was tested (4) within 1 h of sacrifice. Aliquots of 0.05 ml of 10 % homogenates in water were incubated for 5 min at 30° in a final 0.2 ml volume in the presence of 0.25 mg protamin and 40 nanomoles of [γ-^{32}P] ATP.

RESULTS AND DISCUSSION.

At a relatively high concentration (1.10^{-6} M), cyclic AMP was more effective than cyclic GMP in activating basal protein kinase activity in crude

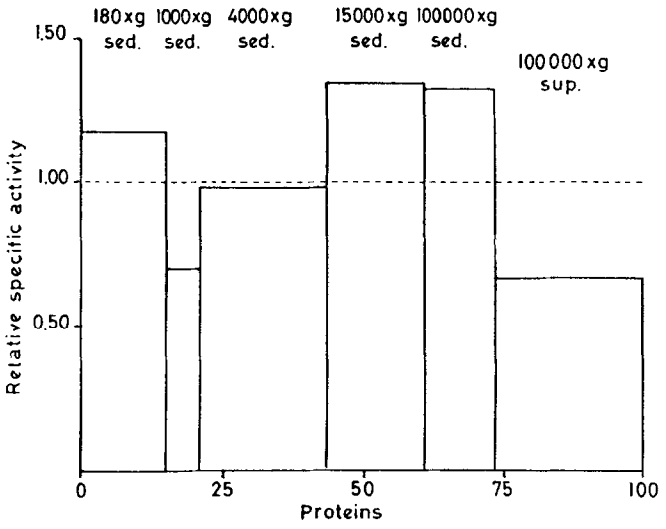

FIGURE 2. DISTRIBUTION OF [^{3}H] CYCLIC AMP BINDING AMONG 6 CRUDE SUBCELLULAR FRACTIONS FROM RAT PANCREAS (Same basic procedure and presentation as in Fig.1). Values are the average of 4 experiments.
Each fraction was incubated at 0° for 100 min in a medium containing 5 µmoles of sodium acetate (pH 4), 0.1 nanomole of [^{3}H] cyclic AMP and 200 µg of bovine serum albumin in a final volume of 0.1 ml by the method of GILMAN (5).

pancreas homogenates. The average increase in activity (in % above basal values ± SEM ; n = 8) was 123 ± 19 % with cyclic AMP, as compared to the lower but still significant increase of 64 ± 10 % with cyclic GMP.

Fourty three per cent of spontaneous kinase activity was found in the 100,000 x g supernatant. The remaining activity was widely distributed among five subcellular particulate fractions. The highest relative specific activity exerted on endogenous substrates was in cytosol and nuclear fractions (Fig. 1). This was also the case in the presence of excess histones (data not shown), indicating that in these fractions, in which there was a high concentration of spontaneous kinase activity, there was a high concentration of endogenous substrates as well.

Fig. 2 shows that the two microsomal fractions and the nuclear pellet exhibited high relative [^{3}H] cyclic AMP binding. This suggests that kinases present in these fractions could still be largely activated by cyclic AMP

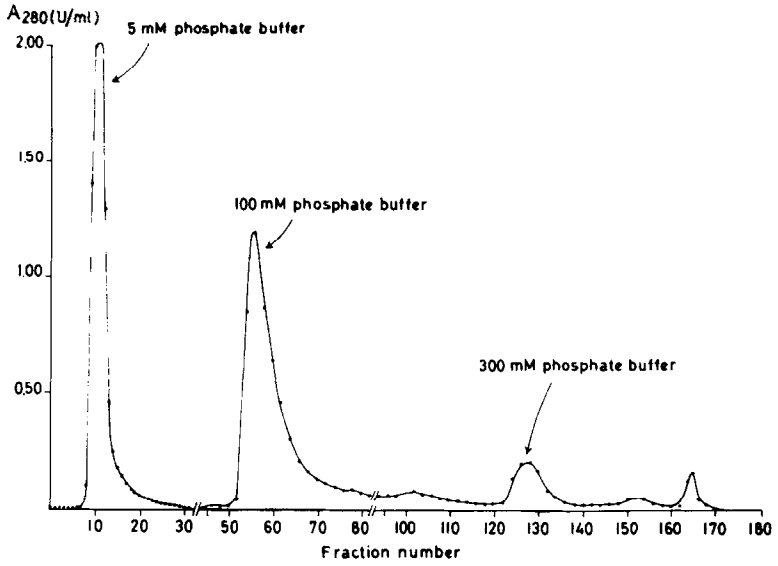

FIGURE 3. SEPARATION OF RAT PANCREAS PROTEIN KINASES BY DEAE-CELLULOSE. Eight rat pancreases were homogenized with 3 volumes of 4 mM EDTA (pH 7) for 2 min in a Waring blender. The homogenate was centrifuged at 27,000 x g for 30 min and the supernatant was adjusted to pH 4.8 with 1 N acetic acid. After centrifugation the acid soluble proteins were readjusted to pH 6.5 with 1 M potassium phosphate buffer pH 7.2 and were submitted to ammonium sulfate precipitation (32.5 g/100 ml). The precipitate was dialyzed against 5 mM potassium phosphate buffer during 14 hours. After dialysis at 4° the enzyme solution was applied to a column of DEAE-cellulose (medium mesh 1 mEq per g) from Whatman (Maidstone, England). The column used (2 x 20 cm) had been washed with 5 bed volumes of 2 mM EDTA-5 mM phosphate buffer (pH 7.0) and the chromatography was conducted with a stepwise phosphate gradient enriched with 2 mM EDTA (pH 7.0). Basal protein kinase activities in pooled fractions from peaks 1, 2 and 3 were, respectively, 23, 69 and 54 fold that observed in the initial homogenate (The phosphorylating activity was tested on histones at a concentration of 2.5 mg/ml).

fixation, while those present in the supernatant were mostly in the active state.

The first steps of protein kinase purification were according to Miyamoto et al. (6) and a DEAE-cellulose chromatography followed (Fig. 3). Protein kinase I eluted with a low phosphate concentration. This relatively cationic and fragile enzyme was not activated by cyclic AMP but was 80 % activated using 1.10^{-6} M cyclic GMP, a concentration decreasing the apparent K_m value for ATP from 0.44 mM to 0.18 mM. Protein kinase II eluted with 100 mM phosphate. This fraction also catalyzed the phosphorylation of histones, but neither cyclic nucleotide stimulated the reaction; rather, a 30 % decrease was observed in the

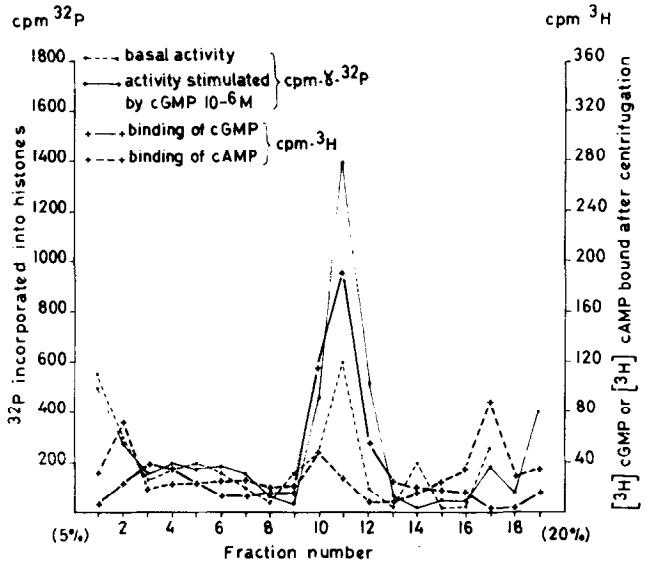

FIGURE 4. SEDIMENTATION PATTERN OF KINASE 1 IN A SUCROSE DENSITY GRADIENT. An aliquot of 0.4 mg of the most active fractions from peak 1 (in Fig. 3) was layered over a 5 to 20 % linear sucrose gradient and centrifuged for 18 h at 49,000 rpm at 0° in a Beckman SW50 rotor. The binding activity was studied in successive fractions on 0.25 ml aliquots as described in Fig. 2. Phosphorylation of added histones (0.5 mg per test) was studied on other aliquots, in the presence and absence of 1.10^{-6} M cyclic GMP under the conditions defined in Fig. 1.

presence of the added nucleotides. This fraction might account for the basal activity observed in the rat pancreas. The last peak, eluted with 300 mM phosphate, was the only one to be markedly (by 210 %) activated by 1.10^{-6} M cyclic AMP, and corresponds to the classical type of kinase containing a catalytic subunit and a regulatory subunit which is able to bind cyclic AMP.

We were interested in studying the sedimentation behavior of protein kinase I. After 18 h of centrifugation, a unique cyclic GMP dependent activity equilibrated in the middle of the sucrose gradient (Fig. 4). This relatively heavy fraction was not only markedly stimulated by 1.10^{-6} M cyclic GMP; but also, it was able to bind [^{3}H] cyclic GMP though not [^{3}H] cyclic AMP. In order to determine if activation by cyclic GMP was accompanied by changes in the sedimentation characteristics, protein kinase I was centrifuged in the presence of 1.10^{-6} M cyclic GMP (Fig. 5). It is clear that a light catalytic sub-

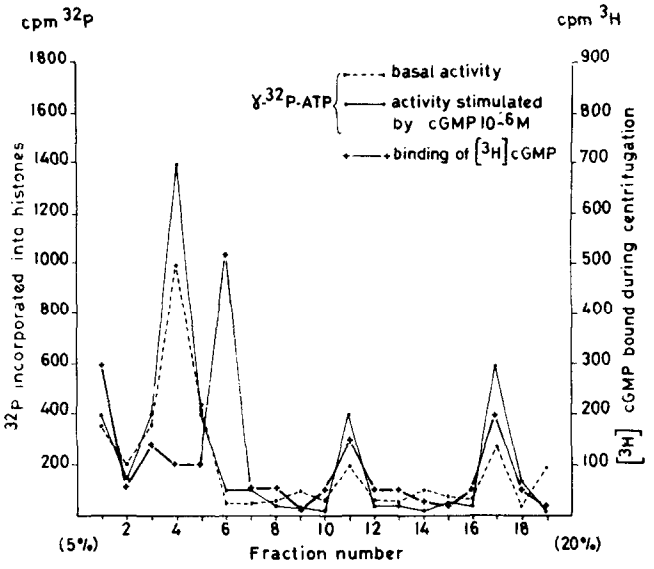

FIGURE 5. SEDIMENTATION PATTERN OF KINASE 1 IN A SUCROSE GRADIENT WITH [^{3}H] CYCLIC GMP IN THE GRADIENT. Same procedure as in Fig. 4 except for the presence of 1.10^{-6} M [^{3}H] cyclic GMP throughout the centrifugation period, and the fact that [^{3}H] cyclic GMP binding was readily tested after filtering 0.1 ml aliquots on Millipore.

unit was able to phosphorylate histones without added cyclic GMP but had no ability to bind cyclic GMP. A somewhat heavier cyclic GMP binding subunit had no capacity for carrying out the phosphorylation of histones. A minor proportion of the original kinase I was not dissociated and kept the position of the complex or aggregated at the bottom of the gradient.

We next studied the affinity of protein kinase I for cyclic GMP. As can be seen from Fig. 6, the double-reciprocal plot was non linear, suggesting the presence of two types of binding sites. The first apparent binding constant of 2×10^{-8} M was lower than the concentration range of cyclic GMP found in the exocrine pancreas ($0.4 - 1.10^{-7}$ M). The second binding constant was more than 10-fold higher. The presence of 5.10^{-8} or 1.10^{-7} cyclic AMP exerted a competitive inhibition on cyclic GMP binding (data not shown).

In mammals so far, only one protein kinase, from rat cerebellum, has been found to be stimulated by cyclic GMP (7). Attempts to separate this kinase by

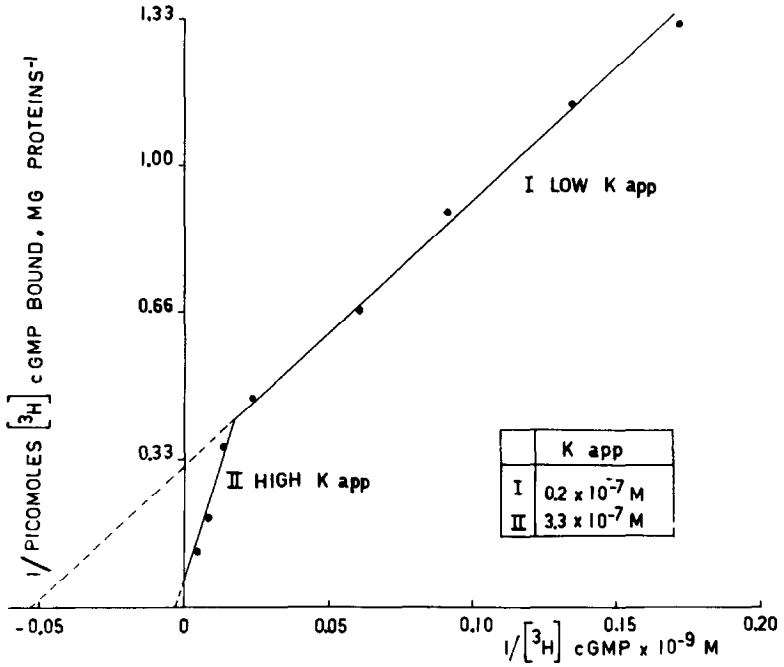

FIGURE 6. BINDING OF CYCLIC GMP BY THE CRUDE 27,000 x g SUPERNATANT OF RAT PANCREAS HOMOGENATES. Two apparent K_a values for cyclic GMP were calculated from the broken double-reciprocal plot. The general incubation conditions were identical to those described in Fig. 2, except for varying cyclic GMP concentrations.

chromatography on DEAE-cellulose resulted in an almost complete loss of the cyclic GMP stimulated activity. In another phyla, a protein kinase from lobster muscle is activated and dissociated by cyclic GMP into a catalytic subunit and a regulatory subunit (8,9).

The present data indicate that the rat exocrine pancreas is well equipped in cyclic AMP and cyclic GMP-dependent kinases. Since acinar cells represent at least 90 % of the gland, we conclude that these cells are responsible for most of the kinase activities. The resting pancreas contains 10 fold higher concentrations of cyclic AMP ($0.4 - 1.10^{-6}$ M) than cyclic GMP ($0.4 - 1.10^{-7}$ M), assuming uniform distribution in intracellular water (unpublished observations). Taking into consideration these basal concentrations, it is clear that full activation of protein kinases can be prevented only by the confinement of a

large proportion of cyclic nucleotides to compartments inaccessible to kinases.

It is acknowledged that attempts to implicate cyclic AMP in enzyme secretion are not yet convincing (references in 1). Pancreozymin -in spite of the presence of a pancreozymin sensitive adenylate cyclase- and carbamylcholine, do not increase cyclic AMP levels in rat pancreas fragments. Urecholine lowers cyclic GMP levels in vitro (Robberecht et al., personal communication), an observation in sharp contrast to increases in cyclic GMP levels reported in brain, heart and thyroid under cholinergic stimulation (11,12). These data on the rat pancreas are consistent with a negative correlation between neurotransmission and guanyl cyclase activity. It appears that if cyclic GMP blocks some effects of cyclic AMP in the pancreas (9), any decrease in cyclic GMP levels would stimulate the activity of cyclic AMP-dependent kinases. This might explain the final similarity of pancreozymin and cholinergic stimulation.

Taken together, our results are compatible with the hypothesis that stimulus-secretion coupling in the exocrine pancreas involves a coordinated monitoring of adenylate cyclase, guanyl cyclase, cyclic nucleotide kinases, and low Km phosphodiesterases (13). Cell compartmentation, the interaction of regulatory and catalytic subunits from several kinases (9), and the presence of an ubiquitous "modulator" (10) could further modulate the proportion of active kinases necessary for the secretory response.

ACKNOWLEDGMENTS.

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