CYCLIC AMP STIMULATED PROTEIN KINASE ACTIVITY WITHIN THE SECRETORY VESICLE FRACTION OF RAT ISLETS

Karl E. Sussman and J. Wayne Leitner

Medical Service, Denver Veterans Administration Hospital and the Division of Endocrinology, Department of Medicine, University of Colorado Medical School, Denver, Colorado 80262

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SUMMARY: The isolated rat islet secretory vesicle fraction (24,000xg) was incubated with gamma [^{32}P ATP] and the TCA-insoluble material was pelleted. The pellet was acid hydrolyzed, lyophilized, redissolved, then subjected to two-dimensional chromatographic separation. Two labeled compounds were identified, i.e., phosphoserine and inorganic phosphorus. With the addition of cyclic AMP (3.5×10^{-6}M), there was a 235% increase in phosphoserine radioactivity ($P<.01$) within endogenous protein subunits. When histones were added to the incubation media, the addition of cyclic AMP resulted in a threefold increase in phosphoserine radioactivity in the TCA-insoluble material ($P<.01$). A comparison was made of cyclic AMP-stimulated protein kinase activity in the homogenate and various islet subcellular fractions. Cyclic AMP-stimulated protein kinase activity is associated with the 24,000xg (secretory vesicle) fraction.

INTRODUCTION: The cyclic AMP activated protein kinase catalyzes the phosphorylation of the amino acids serine or threonine within protein subunits of the cell. The accepted proof of the existence of protein kinase activity in cells or subcellular organelles is to demonstrate phosphorus labeling of endogenous serine and/or threonine residues following incubation with labeled ATP.[1] If histones are added as a phosphate receptor, it is technically easier to demonstrate cyclic AMP-stimulated protein kinase activity.

Upon incubation of the secretory vesicle fraction of rat islets with labeled ATP, radioactive ADP, AMP and cAMP are generated.[2] Concomitant with this conversion, the water insoluble constituents of the secretory vesicle fraction become labeled with phosphorus that cannot be accounted for by the presence of adenine nucleotides per se. This phosphorylation is stimulated by the addition of cyclic AMP.[2]

The purpose of the present series of experiments is to determine if a cyclic AMP-stimulated protein kinase is present within the islet secretory vesicle

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fraction, specifically to examine phosphorus labeling of serine within the insoluble constituents in the presence of cyclic AMP.

MATERIALS AND METHODS: Animals: Male Sprague Dawley rats weighing 250-300 gms were obtained from the Simonson Laboratories, Gilroy, California. The animals were allowed food and water ad libitum. Anesthesia was induced with pentobarbital (Diabutal), 45 mg/kg body weight given intraperitoneally.

Materials: Collagenase (CLS IV, Lot #45K152S, 202 units/mg) was obtained from the Worthington Biochemical Corporation, Freehold, N.J. Gamma [^{32}P] ATP (S.A. 28.29 Ci/mmole) and PCS solubilizer were obtained from Amersham Searle Corporation, Arlington Heights, Ill. Pre-coated plates for thin layer chromatography (20x20 cm, layer: 0.1 mm Cellulose MN300) were obtained from Brinkman Instruments, Inc., Des Plaines, Ill. Biochemicals were obtained from the Sigma Chemical Company, St. Louis, Mo. RP X-Omat medical X-ray film was supplied by the Eastman Kodak Company, Rochester, N.Y.

Methods: Isolation of subcellular fractions: Rat islets were isolated by the method of Lacy and Kostianovsky with the modification of Leitner, et al.[3,4] The secretory vesicle fraction (24,000xg) was prepared according to the method of Leitner, et al. which was a modification of the original technique of Howell, Fink and Lacy.[4,5]

The 24,000xg fraction is rich in insulin (the richest if the insulin level is compared relative to protein content of each subcellular fraction).[2] This fraction contains no measurable acid phosphatase, low levels of RNA (5.3% of that found in the homogenate), cytochrome C reductase (3.2%), 5' nucleotidase (5.4%) and DNA (8.4%). It had been previously demonstrated that this same fraction contained relatively little cytochrome oxidase.[4] The relative purity of this fraction is discussed elsewhere.[2,4,5]

Radioactive incubation and then layer chromatography: The secretory vesicle fraction (containing 13.12 $\pm$ 1.21 µg of protein) was suspended in 0.10 ml of 0.30 M sucrose in 5mM phosphate buffer, pH 6.0. To this was added 0.10 ml of reaction medium consisting of 100mM acetate buffer, pH 6.0; 20mM magnesium chloride; 40mM sodium fluoride; 10mM theophylline; 600 µM EGTA; 30 µM antimycin A; 8.26 µCi of ATP labeled with [^{32}P] in the gamma (terminal) phosphorus position. In certain designated experiments, the reaction mixture contained 7.0 µM cAMP and/or 1.00 mg/ml histone, resulting in a final incubation medium with 3.5 µM cAMP and/or 100 µg of histone. The secretory vesicle fraction was incubated at 30C for ten minutes. The reaction was terminated by placing the incubation tubes in a water bath at 100C for three minutes. This is a standard incubation procedure which has been utilized to demonstrate protein kinase activity.[6-10]

Four volumes of 10% TCA at 2C were added to the incubation tubes and the acid insoluble material was pelleted at 106,000xg for 60 minutes. The supernatant was removed and the pellet fraction was washed three times with 2.0 ml of 6% TCA at 106,000xg for 30 minutes.

The pellet was then dissolved in 6.0 N HCl and placed in a 1.0 ml ampule. Ten microliters of 40mM phosphoserine and inorganic phosphorus were added as carrier standards. The ampule was flame-sealed and the pellet material was hydrolyzed by placing the tightly sealed ampule in a water bath at 100C for four hours. Under these stated conditions of hydrolysis, 28% of the phosphoserine undergoes cleavage with resultant loss of phosphorous. In contrast, 86% of the histone material was hydrolyzed to its component amino acids. These studies were performed using calf thymus Type II-A histones (98.36 µg) and O-phospho-L-serine (14.5 µg which is equivalent to 2.4 µg phosphorous).

The hydrolyzed material was lyophilized to dryness and then reconstituted with 100 µl distilled water. Sixty microliters of this material were spotted on thin layer chromatography plates and subjected to two-dimensional chromatographic

TABLE I

R_f Values for Phosphorylated Compounds Being Separated by Thin Layer Chromatography

	R_f [1]	R_f [2]
Phosphoserine	0.391	0.276
Inorganic Phosphorous	0.555	0.605
AMP	0.464	0.270
ADP	0.285	0.197
ATP	0.186	0.094
cAMP	0.426	0.082

1. Phase I for 2 hours in t-pentyl alcohol, formic acid and water (3:2:1).

2. Phase II for 3 hours in t-pentyl alcohol and water (2:1) with 6 gms p-toluenesulfonic acid.

separation. The first phase consisted of t-pentyl alcohol, formic acid and water in a proportion of 3:2:1. Phase I was run for 2 hours after the chromatography tank had been equilibrated with the solvent system for 1 hour. Phase II consisted of 180 ml t-pentyl alcohol and 90 ml water containing 6 gms p-toluenesulfonic acid. The second phase was run for 3 hours after the equilibration period. The plates were dried for an interval of 18 hours between the two phases.

To identify the phosphate compounds of interest, the plates were sprayed with a ammonium molybdate solution (60 ml water, 5 ml 60% perchloric acid, 10 ml 1.0 N HCl, and 25 ml 4% ammonium molybdate). An area of 2.6 x 5.1 cm containing and surrounding the spots was removed and placed in a liquid scintillation cocktail (PCS) for radioactive counting. Appropriate control samples (containing no secretory vesicle material) were carried through the entire radioactive incubation, acid hydrolysis, chromatographic and scintillation procedures.

For autoradiography, twenty microliters of resuspended hydrolysate were applied to the plates and the plates subjected to chromatographic separation as described above. The chromatographs were placed in contact with X-ray film for 14 days and the film subsequently developed.

RESULTS: In the present study, samples of TCA insoluble secretory vesicle material were hydrolyzed and then subjected to thin layer chromatography. This chromatographic system allows the separation of phosphoserine and inorganic phosphate as well as various adenine nucleotides (Table I). Heretofore, high voltage electro-

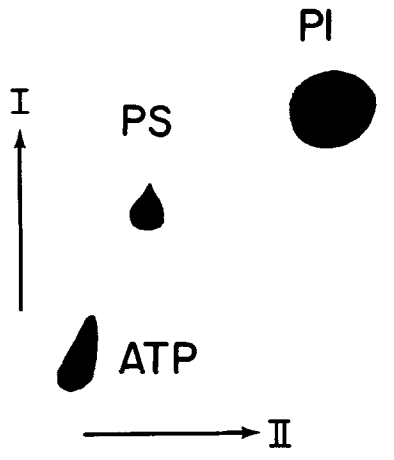
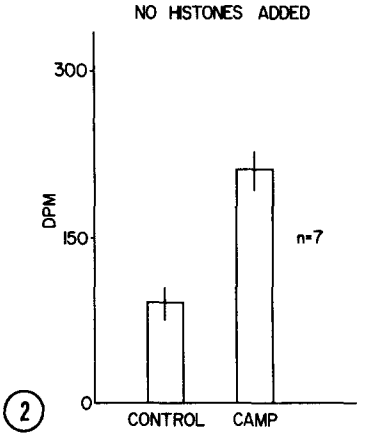


Figure 1 Separation of ATP, phosphoserine and inorganic phosphate standards employing thin layer chromatography. When a sample under study is subjected to acid hydrolysis, the ATP undergoes cleavage and only two compounds are found, phosphoserine and inorganic phosphate.

phoresis has been employed to separate phosphoserine and inorganic phosphorus for the assay of protein kinase.

Figure 1 demonstrates the separation of ATP, phosphoserine, and inorganic phosphate carriers on thin layer chromatography. When the sample is subjected to acid hydrolysis, the ATP undergoes cleavage with the formation of inorganic phosphate. Thus, only two compounds, phosphoserine and inorganic phosphate, are recovered. Autoradiography confirms that only these two labeled compounds are present. If substantially more radioactivity is employed than is used in the present study, phosphothreonine can also be identified located between phosphoserine and inorganic phosphate. This is more apt to occur if the incubations are performed in the presence of histones with cyclic AMP added.

Figure 2 shows phosphorus radioactivity in serine residues in the insoluble secretory vesicle material which has been subjected to acid hydrolysis. In the endogenous protein material, the addition of cyclic AMP increased phosphoserine radioactivity from 93 $\pm$ 12 to 219 $\pm$ 18 dpm ($P<.01$). In the presence of added



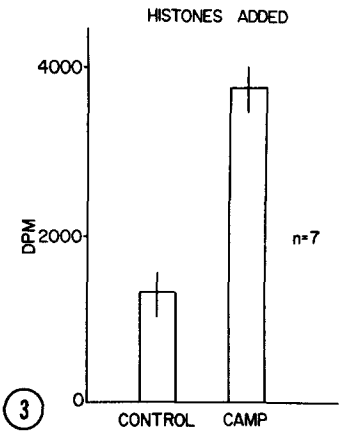


Figure 2 Increase in ^{32}P phosphoserine radioactivity in the insoluble secretory vesicle material with the addition of cyclic AMP (3.5×10^{-6}M). Protein content is 13.12 ± 1.21 µg. Shown are the mean values ± SEM.

Figure 3 Increase in ^{32}P phosphoserine radioactivity in the insoluble secretory vesicle material with the addition of cyclic AMP (3.5×10^{-6}M). Histones (100 µg) have been added to act as a phosphate receptor. Vesicle protein content is 13.12 ± 1.21 µg. Shown are the mean values ± SEM.

histones (Fig. 3), the increase in phosphoserine radioactivity with cyclic AMP was from 1,281 ± 317 to 3,864 ± 657 dpm ($P<.01$).

Figure 4 shows a comparison made of cyclic AMP-stimulated protein kinase activity in the islet homogenate and various subcellular organelles. These incubations were performed in the presence of added histones. The actual control and stimulated levels of phosphoserine radioactivity are given in Table II. The 24,000xg fraction (thought to represent predominately secretory vesicle material) contains 18.12 ± 3.45% of the total cyclic AMP-stimulated protein kinase activity measured in the four subcellular fractions.

DISCUSSION: Cyclic AMP has been shown to stimulate insulin secretion, and there has been considerable focus on the role of cyclic AMP in insulin release induced by a variety of secretagogues.[11-16] Although the importance and the precise role of cyclic AMP in augmenting insulin secretion is as yet unknown,

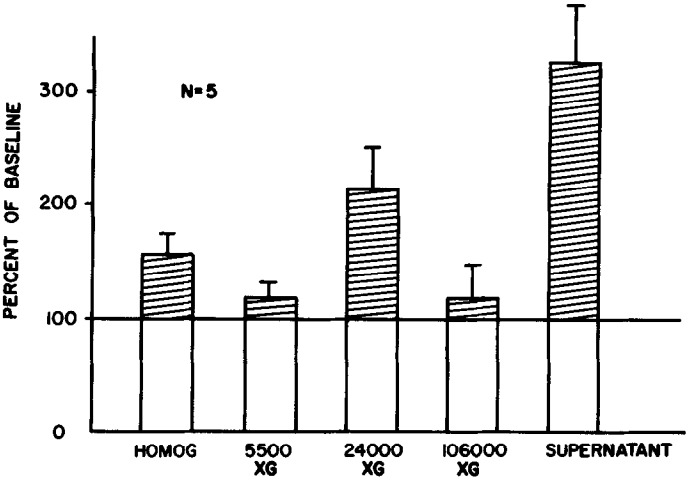


Figure 4 Comparison of the levels of cyclic AMP stimulated protein kinase activity within the homogenate and various subcellular organelles. The hatched area indicates the percent increase in phosphoserine radioactivity above baseline with the addition of cyclic AMP (3.5×10^{-6}M). Shown are the mean values ± SEM.

few will deny that cyclic AMP may have a primary role in insulin release induced by certain secretagogues. Cyclic AMP generation may be just one step in a cascade of metabolic events occurring as insulin is released.[17]

The present data suggest that cyclic AMP stimulated protein kinase activity is associated with the secretory vesicle fraction (24,000xg) of rat islets. Dods and Burdowski observed protein kinase and phosphoprotein phosphatase activities in rat islets of Langerhans.[18] Montague and Howell have shown that various insulin secretagogues stimulate insulin release along with increasing islet protein kinase activity.[19]

It seems reasonable to suggest that there may be protein kinase activity within various subcellular organelles which will permit the cell to engage in discrete functions at any one moment in time (e.g. membrane transport, genetic message transmission, various secretory phenomena etc). It is not unexpected therefore that protein kinase activity is found in association with the secretory

TABLE II

Protein Kinase Activity in Islet Subcellular Fractions

	Protein (μg)	- Cyclic AMP		+ Cyclic AMP		Cyclic AMP-stimulated protein kinase activity (Δdpm)	Ratio $\frac{+cAMP}{-cAMP}$
		Total Activity[a] (dpm)	Activity/μg of protein[b] (dpm/μg)	Total Activity[a] (dpm)	Activity/μg of protein[b] (dpm/μg)		
Homogenate	55.11 ± 5.17	5062 ± 515	110 ± 14	8233 ± 924	175 ± 24	3171 ± 447	1.580 ± 0.102
5,500xg	26.93 ± 1.73	2434 ± 358	87 ± 9	2898 ± 473	108 ± 13	465 ± 135	1.217 ± 0.081
24,000xg	13.63 ± 1.23	1102 ± 176	88 ± 12	2140 ± 307	183 ± 25	1038 ± 139	2.121 ± 0.175*
106,000xg	6.51 ± 1.15	883 ± 160	95 ± 8	957 ± 202	102 ± 13	74 ± 14	1.122 ± 0.221
Supernatant from 106,000xg	10.93 ± 1.49	1997 ± 317	224 ± 40	6466 ± 794	677 ± 104	4469 ± 517	3.367 ± 0.316*
% Recovery of total present in homogenate	104.76 ± 2.65	123.92 ± 9.80		154.88 ± 9.56			

[a]Activity is defined as the ^{32}P radioactivity recovered at the phosphoserine locus following acid hydrolysis and thin layer chromatography. This represents the ^{32}P incorporated in the serine residues of 100 μg histone per 10 minutes at pH 6.0.

[b]This is an expression of the protein kinase activity in terms of the ^{32}P phosphorylation of histone serine residues per μg of subcellular protein.

*$p < .01$

vesicle fraction of rat islets. Labrie and co-workers similarly have reported cyclic AMP-dependent protein kinase activity within isolated adenohypophyseal secretory granules.[20] Muller and associates measured the effect of cyclic AMP on [^{32}P] incorporation into TCA precipitable aliquots of various islet subcellular fractions and suggested the presence of cyclic AMP-stimulated protein kinase activity in all fractions.[21]

Sharp and co-workers were able to demonstrate islet protein kinase activity in the presence of histones but did not observe any phosphorylation of endogenous islet protein.[17] This same group suggested that the major site of action of cyclic AMP may be on the phosphoprotein phosphatase.

Utilizing markers for subcellular organelles, such as 5'-nucleotidase and NADPH-cytochrome C reductase, it appears that membrane or microsomal contamination could not account for the levels of cyclic AMP-stimulated protein kinase activity observed in the secretory vesicle fraction.[2]

It is apparent that the activity reported represents net phosphorylation, that is, the combined effects of protein kinase and phosphoprotein phosphatase activities. In addition, there may have been present in the designated subcellular fractions, varying amounts of the thermostabile inhibitor of protein kinase. Thus, although the evidence is persuasive that cyclic AMP-stimulated protein kinase activity is an integral part of the secretory vesicle, this statement cannot be made without reservation.

In this report and the others previously noted, basal phosphorylation in the absence of cyclic AMP has been consistently observed. This suggests that there may be present in the secretory vesicles and in other subcellular fractions a protein kinase which is not dependent on the presence of cyclic AMP. Alternatively, it is conceivable that there may be some cyclic AMP present, either as an integral constituent of subcellular organelles or that which has co-precipitated with the granules during the process of isolation and subcellular fractionation. For the moment, there is no clear explanation for the observed basal protein phosphorylation.

The present data suggest that cyclic AMP stimulated protein kinase activity appears to be an integral part of the secretory vesicle fraction of rat islets. The precise role of this particular protein kinase activity in insulin biosynthesis and/or secretion remains to be elucidated.

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