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PROTEIN PHOSPHORYLATION IN PANCREATIC ISLETS: EVIDENCE FOR SEPARATE Ca^{2+} AND cAMP-ENHANCED PHOSPHORYLATION OF TWO 57,000 Mr PROTEINS

Anjaneyulu Kowluru and Michael J. MacDonald*

Department of Pediatrics
University of Wisconsin School of Medicine
1300 University Avenue
Madison, Wisconsin 53706

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SUMMARY: There is a phosphopeptide that has an Mr of 53,000 to 60,000 in insulin-secreting tissues and there is general agreement that this peptide can be phosphorylated in a calcium-dependent manner. The present report shows that there are at least two phosphoproteins with Mr's near 57,000 in rat pancreatic islet cytosol. One peptide has an Mr of 57,000, a pI of 7.5 - 8 and is phosphorylated in a Ca^{2+}-enhanced manner, and the other has an Mr of 54,000, a pI of 5 - 5.5 and is phosphorylated in a cAMP-enhanced manner, as judged by two-dimensional polyacrylamide gel electrophoresis. Sepharose 4B chromatography indicated that the former polypeptide resides in a native protein complex that has an Mr of about 500,000 and the latter in a complex that has an Mr of about 180,000. Tritiated azido cyclic AMP binds to an islet polypeptide that has an Mr of 54,000. The results suggest that Ca^{2+} and cAMP could regulate stimulus-secretion coupling in pancreatic islets via protein phosphorylation.

Insulin secretion by pancreatic islets is known to be associated with a concomitant increase in intracellular calcium (1) and it is widely believed that calcium is a messenger for stimulus-secretion coupling in the beta cell. Because one of the effects of calcium in brain, muscle and platelets is to stimulate protein phosphorylation, particularly via calmodulin, it is logical to look for calcium-activated protein phosphorylation in pancreatic islets. Islets have been shown to have calmodulin and furthermore, calmodulin inhibitors, such as trifluoperazine (2,3) and W-7 (4) can inhibit insulin secretion.

At least two calcium-calmodulin activated protein kinases in pancreatic islets have been reported. One is myosin light chain kinase (5) and the other is a kinase that catalyzes the phosphorylation of a peptide with an

*To whom correspondence should be addressed.

ABBREVIATIONS: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(Nmorpholino)-propanesulfonic acid; SDS, sodium dodecyl sulfate.

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Mr of 53,000 to 60,000. The latter phosphopeptide has been widely described in insulin-secreting tissues (6-9) and its phosphorylation is inhibited by trifluoroperazine. Phosphorylation of this polypeptide has been speculated to be stimulated by both calcium and cAMP (8). However, in the present report we show that there are two islet cytosol polypeptides with Mr's near 57,000. Phosphorylation of one polypeptide is enhanced by calcium and phosphorylation of the other polypeptide is enhanced by cAMP.

EXPERIMENTAL PROCEDURES

Pancreatic islet isolation. Islets were isolated from pancreata of well fed 250 g Sprague-Dawley rats by collagenase digestion of the pancreata (10) and centrifuging the digested pancreata in a discontinuous gradient of Ficoll (11). The islets in the upper layers of the Ficoll gradient were then picked manually with siliconized glass pipettes. Only islets that were completely free of acinar and connective tissue were selected (12,13).

Cytosol preparation. Islets were washed once in 0.23 M mannitol, 0.07 M sucrose, 5 mM Hepes buffer, pH 7.5, 1 mM dithiothreitol and 1 mM EGTA and then twice in the same solution not containing EGTA. Approximately 1500 islets (about 0.6 mg protein) were homogenized in 0.2 ml of the sucrose-mannitol solution without EGTA and centrifuged in a 1.4 ml polypropylene Eppendorf centrifuge tube at 105,000 xg for 1 h in a Beckman 40 rotor, type d. Cytosol was the supernatant fraction (12,13). All procedures were done at 4°.

Protein phosphorylation. For studying protein phosphorylation, islet cytosol was incubated at 37° in a final volume of 40 µl containing 10 mM magnesium acetate, 1 mM dithiothreitol, 50 mM MOPS buffer, pH 7.0, 25 µM {γ-^{32}P}ATP (2,500 cpm/pmol) and, unless stated otherwise, 100 µM $CaCl_2$ and 150 nM added calmodulin. The reaction was initiated by adding ATP and stopped by heating at 100° for 2 min in electrophoresis sample solution containing 1% SDS. Electrophoresis in 0.1% SDS in 10% polyacrylamide slab gels (stacking gels 3% polyacrylamide) was done by the method of Laemmli (14). The gels were then stained in 0.4% Coomassie brilliant blue R to identify the molecular weight marker proteins. The gels were then exposed to X-Omat AR film (Eastman Kodak Company) and Cronex Lightning Plus intensifying screens (E. I. Dupont De Nemours Company) (15) at -70° for 12 to 48 h to demonstrate ^{32}P incorporation into proteins. The densities of the radiolabelled bands were measured by laser densitometry (LKB Model 2201 Laser Densitometer) and by integrating the areas under the densitometric tracings. Proteins with the following subunit Mr's were used to calibrate molecular weight: rabbit skeletal muscle phosphorylase a (92,500), pyruvate kinase (57,000), actin (42,000) and aldolase (40,000), cow's milk lactoperoxidase (82,000), bovine serum albumin (68,000), pig heart fumarase (38,000), bovine pancreas α-chymotrypsinogen (25,000) and horse heart myoglobin c (17,200). ^{32}P labelled islet cytosol proteins were also separated by two-dimensional polyacrylamide gel electrophoresis according to the method of O'Farrell (16). The first dimension was isoelectric focusing nominally between 5 and 7.5 in 8 M urea in disk gels and the second dimension was electrophoresis in SDS slab gels described above. Companion disk gels were cut into 0.5 cm lengths, pulverized and placed in degassed water for pH measurements to calibrate the first dimension.

Sepharose 4B chromatography of phosphorylated proteins. Approximately 200 µg of cytosol protein was incubated in the phosphorylation assay mixture as described above in the presence of either 100 µM $CaCl_2$ plus 150 nM added cal-

modulin or 10 mM EGTA alone or 10 mM EGTA plus 30 µM dibutyryl cAMP. The reaction was stopped after 10 min by adding sodium fluoride, Tris chloride buffer, pH 7.5, NaCl sodium pyrophosphate, and $MgCl_2$ to give final concentrations of 100 mM, 15 mM, 0.6 M, 0.5 mM and 0.5 mM, respectively, and immediately applying the sample (60 µl) to a 6.3 cm x 0.13 cm^2 Sepharose 4B column. The column was previously equilibrated with 15 mM Tris chloride buffer, pH 7.5, 0.6 M NaCl, 0.5 mM sodium pyrophosphate, 0.5 mM $MgCl_2$ and 2 mM dithiothreitol. Fifty microliter fractions were collected directly into 10 µl of SDS to give a final dodecyl sulfate concentration of 1%. The fractions were immediately heated at 100° and applied to polyacrylamide gels as described above. The column void volume and salt peak were estimated with dextran blue and sodium azide and the elution positions of Mr marker proteins were estimated by measuring their enzyme activities (17).

Photoaffinity labelling. This was carried out by the procedure reported by Walter et al (18,19) with minor modifications. Islet cytosol was incubated in a total volume of 80 µl containing 50 mM MOPS buffer, pH 6.8, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM isobutylmethylxanthine, 0.5 mM β-mercaptoethanol and 1 µM {2-^{3}H(N)} 8-azidoadenosine 3'5'-cyclic phosphate (NH_4 salt, 16 Ci/mmol). The reaction was carried out with and without added unlabelled cAMP (10 µM) at room temperature for 5 minutes in the dark. The tubes were then cooled in ice water and photolysis was performed in Kimax glass tubes (50 mm X 6 mm, 1 mm thickness) with a 1 KW AH-6 high pressure mercury lamp (Advanced Radiation, Santa Clara, CA) for 3 sec at 10 cm distance from the lamp. (The lamp output was 29.8 W at 220-280 nm, 69.5W at 280-320 nm, 112.3W at 320-400 nm and 252.3W at 400-760 nm.) After photolysis, the samples were subjected to SDS-polyacrylamide gel electrophoresis. Gels were treated with En^{3}Hance (New England Nuclear) and autofluorography was carried out as described above.

Materials. Na_3ATP was from P-L Biochemicals. Bull testes calmodulin was a gift of Dr. Frank Siegel. Sepharose 4B was from Pharmacia and highly purified Tris was from Boehringer-Mannheim. {γ-^{32}P}ATP and {2-^{3}H(N)} azido cAMP were from Amersham. All other chemicals were from Sigma in the highest purity available.

RESULTS AND DISCUSSION

The calcium-dependent nature of the phosphorylation of the islet protein with an Mr of 53,000 to 60,000 has been well described and its phosphorylation is inhibited by trifluoperazine (6-9), suggesting that the phosphorylation is calmodulin-dependent as well as calcium-dependent. Harrison and Ashcroft (8) found that the Mr of the calcium-dependent phosphopeptide was 53,100 and that a polypeptide with a similar Mr underwent cAMP-dependent phosphorylation. They speculated that the polypeptide substrates for calcium and for cAMP-dependent phosphorylation were one and the same polypeptide. Their conclusion was based on a one-dimensional SDS-polyacrylamide gel electrophoresis study.

Previous work suggested that there may be two islet phosphopeptides with Mr's in the range of 54,000 to 57,000 (7). One polypeptide has a pI of about 7.5, whereas the other has a pI of 5 - 5.5., as judged by two-

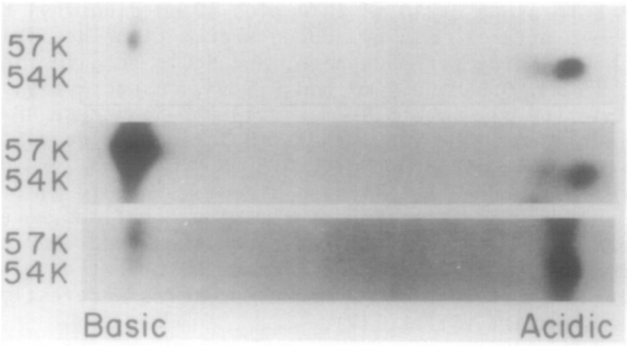

Figure 1: Effect of EGTA, Ca^{2+} or cAMP on phosphorylation of pancreatic islet cytosol proteins with Mr's near 54,000-57,000. The figure shows portions corresponding to an Mr near 54,000-57,000 of autoradiograms of three two-dimensional polyacrylamide gels. Pancreatic islet cytosol was incubated with {γ-^{32}P}ATP and either 10 mM EGTA (uppermost panel), 100 µM $CaCl_2$ and 150 nM added calmodulin (middle panel) or EGTA and 30 µM dibutyryl cAMP (lowest panel) and then the proteins were separated by two-dimensional electrophoresis and identified by autoradiography. A linear pH range of 5.2 to 7.5 was used. The autoradiograms are depicted with isoelectric focusing in the horizontal dimension and electrophoresis in SDS in the vertical dimension.

dimensional gel electrophoresis. A more thorough study of this question is described in this communication. Figure 1 shows portions of autoradiograms corresponding to the 54,000 - 57,000 Mr range of three two-dimensional SDS-polyacrylamide electrophoresis gels. Phosphorylation of the basic polypeptide was greater in the presence of Ca^{2+} (middle panel) than in the presence of EGTA alone (top panel) or EGTA plus dibutyryl cAMP (bottom panel), whereas phosphorylation of the acidic polypeptide was greatest in the presence of dibutyryl cAMP.

Another indication that islet cytosol contains two phosphorylatable polypeptides with Mr's in the range of 54,000 to 57,000 comes from the experiments depicted in Figures 2-4. Two equal portions of islet cytosol were incubated with {γ-^{32}P}ATP and either Ca^{2+} or EGTA plus cAMP and then quickly applied to Sepharose 4B columns. A protein with a subunit Mr of 57,000 that was phosphorylated in a Ca^{2+}-enhanced manner eluted from the column closer to the void colume than a protein with a subunit Mr of 54,000 that was phosphorylated in a cAMP-enhanced manner. The Mr's of the native protein complexes, as estimated from the Sepharose chromatography were 500,000 for the one phosphorylated in a Ca^{2+}-enhanced manner and 180,000 for one phosphorylated in a cAMP-enhanced manner.

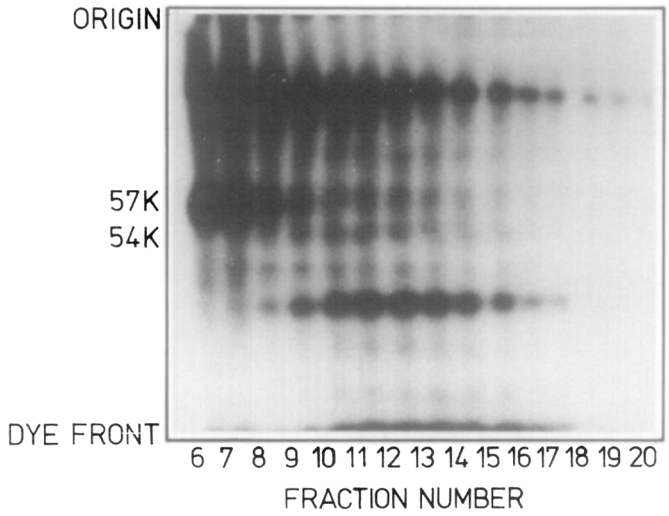

Figure 2: SDS polyacrylamide gel electrophoresis and autoradiography of pancreatic islet cytosol after incubation with {γ-^{32}P}ATP and Ca^{2+} and fractionation on Sepharose 4B. One-half of a sample of islet cytosol was incubated with 100 µM Ca^{2+} and 150 nM calmodulin and ATP immediately applied to a Sepharose 4B column as described under Experimental Procedures. Fractions were collected in SDS, heated immediately after elution from the column and subjected to SDS gel electrophoresis. The other half of the cytosol was used in the procedure depicted in Figure 3.

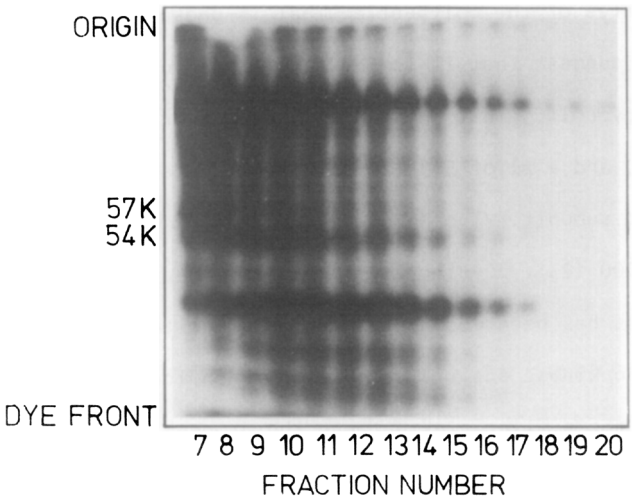

Figure 3: SDS polyacrylamide gel electrophoresis and autoradiography of pancreatic islet cytosol after incubation with {γ-^{32}P}ATP and cAMP plus EGTA. The other half of the same sample of cytosol used in the experiment depicted in Figure 2 was incubated with 30 µM dibutyryl cAMP, 10 mM EGTA and ATP and fractionated on a Sepharose 4B column. Conditions were identical to those used in the procedure depicted in Figure 2 and the procedure was carried out almost simultaneously with that depicted in Figure 2.

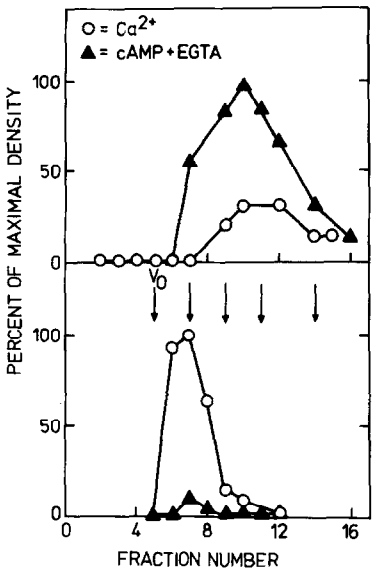

Figure 4: Effect of Ca^{2+} and cAMP on elution profiles of the phosphoproteins with 54,000 and 57,000 from Sepharose 4B columns. The autoradiograms depicted in Figures 2 and 3 were scanned with a laser densitometer and the areas underneath the tracings of the 54,000 Mr bands (upper panel) and the 57,000 Mr bands (lower panel) were plotted vs. the fraction numbers. The results are expressed as a percent of the maximal density of each band. The arrows indicate, from left to right, the positions of the column void volumes (Vo) and the elution positions of peak activity of standard proteins with the following Mr's: rabbit skeletal muscle myosin (440,000), pyruvate kinase (237,000), and lactate dehydrogenase (144,000) and pig heart malate dehydrogenase (74,000). The column salt peak was in fraction 22.

The properties of the polypeptide that is phosphorylated in a cAMP-enhanced manner suggest that it is the regulatory subunit of cAMP-dependent protein kinase type II. This subunit has been reported to have an Mr of 54,000 to 56,000 and a pI of 5.3 (20-23) and it undergoes autophosphorylation by its catalytic subunit. The Mr of the holoenzyme from brain and heart is 176,000 to 177,000 (23). Cyclic AMP-dependent protein kinase activity in pancreatic islets has been documented (24,26), but the type(s) of cAMP-dependent protein kinase activities have not been identified.

In view of the similarities between the properties of the islet polypeptide phosphorylated in a cAMP-enhanced manner and cAMP-dependent protein kinase type II, islet cytosol was incubated with tritiated azido cAMP in an attempt to label islet proteins by a photoaffinity technique. Figure 5 shows that an islet polypeptide with an Mr of 54,000 was the most prominently labelled islet polypeptide. The labelling is apparently specific because

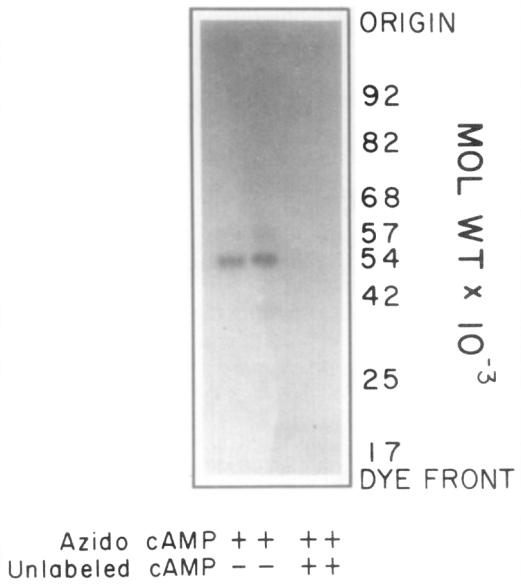

Figure 5: Photocatalyzed labeling of pancreatic islet cytosol proteins with tritiated azido cAMP. Islet cytosol was incubated with tritiated azido cAMP in the presence and absence of unlabeled cAMP (20 μM), photolysis was carried out, and the sample applied to an SDS polyacrylamide gel as described under Experimental Procedures.

it was prevented by adding unlabelled cAMP. Although it cannot be known with certainty that the polypeptide with an Mr of 54,000 that binds azido cAMP is the same 54,000 Mr polypeptide that is phosphorylated in a cAMP-enhanced manner, taken together the results reported herein are consistent with this idea.

No one has suggested a plausible identity for the 57,000 Mr protein that is phosphorylated in a calcium-enhanced manner. A suggestion that it is the α and β subunits of tubulin (27) does not appear tenable, since phosphorylated tubulin has a pI of 5.2 - 5.5 (28,29), whereas the 57,000 Mr protein in islet cytosol that is phosphorylated in a calcium-enhanced manner has a pI of about 7.5 (ref. 7 and Figure 1). Moreover, others have reported that the phosphoprotein in question is definitely not tubulin (30).

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