Phosphorylation of parathyroid secretory protein

(calcium/regulation/hormone/protein kinase)

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ABSTRACT The phosphorylation of proteins released into the medium of bovine parathyroid gland slices or isolated cells incubated with ³²P_i has been investigated. The primary protein phosphorylated had a *M_r* of 68,000 and coeluted with newly synthesized parathyroid secretory protein (PSP) on Bio-Gel chromatography and on polyacrylamide gel electrophoresis. Isoelectric focusing of double-labeled samples ([³⁵S]methionine and ³²P_i) revealed comigration of the two radioactive markers at a pH of 4.6, which was similar to that of purified PSP. Phosphorylation of the *M_r* 68,000 protein was also demonstrated in cell homogenates incubated with [³²P]ATP; the *M_r* 68,000 protein was the predominant labeled protein. Increasing quantities of calcium, with and without added EGTA, caused a progressive decrease in phosphorylation of the protein. These studies demonstrate that PSP is readily phosphorylated in parathyroid cells, that the degree of phosphorylation is inversely proportional to calcium concentration, and that PSP is the major phosphorylated protein released from the gland. The relationship of phosphorylation to the potential physiologic importance of PSP remains to be determined.

Parathyroid secretory protein (PSP) is a high molecular weight glycoprotein of unknown physiologic function that is secreted by the parathyroid gland (1–4) and has recently been purified from bovine parathyroid glands (5, 6). Like parathyroid hormone (PTH), its secretion is inversely proportional to extracellular calcium concentration (7–9), with PSP and PTH accounting for 50% and 20% of the newly synthesized protein released by the gland (1). The mechanism by which calcium regulates the secretion of these two proteins is under active investigation. A general mechanism for regulation of protein phosphorylation by calcium has been suggested in other tissues and appears to involve calmodulin-dependent protein kinase activity that catalyzes the phosphorylation of endogenous substrates (10, 11).

In the present study, we examined the phosphorylation of endogenous proteins in the parathyroid gland. We report here on the phosphorylation of an endogenous protein of *M_r* 68,000 that is actively released by the gland and is indistinguishable from PSP.

MATERIALS AND METHODS

Materials. Carrier-free ³²P_i or [³²P]ATP (10 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ Bq) and L-[³⁵S]methionine (940 Ci/mmol) were obtained from Amersham. Ampholytes (pH 3.5–7.0) were obtained from LKB. All chemicals used in column chromatography and gel electrophoresis including *M_r* standards were from Bio-Rad.

Methods. Fresh bovine parathyroid glands were obtained from the slaughterhouse and kept on ice. They were trimmed of fat and then slices were prepared with a Stadie–Riggs tissue slicer. The slices were incubated with ³²P_i (100 μCi/ml) with

and without [³⁵S]methionine (10 μCi/ml) in a shaking water bath for 2–4 hr at 37°C in phosphate-free Krebs–Ringer bicarbonate buffer (KRB buffer; 120 mM NaCl/4.75 mM KCl/1.2 mM MgCl₂/0.5 or 2.5 mM CaCl₂/25 mM NaHCO₃, pH 7.4, containing 2 mg of glucose per ml) in an atmosphere of 95% O₂/5% CO₂. At the end of the incubation period, the medium was separated from tissue by centrifugation at 650 × *g* for 10 min in a refrigerated centrifuge. Medium was then centrifuged at 8,500 × *g* for 20 min and used for further studies. Isolated cells were prepared by the method of Brown *et al.* (12) and were incubated with ³²P_i in phosphate-free KRB buffer. Cells and media were then processed for NaDodSO₄/polyacrylamide gel electrophoresis or column chromatography on Bio-Gel A-1.5m (1.2 × 75 cm) in 0.15 M ammonium bicarbonate pH 7.8 buffer. After column chromatography, the radioactive peak fractions were pooled, lyophilized, and analyzed further.

For polyacrylamide gel electrophoresis, the proteins in the medium were precipitated by addition of an equal volume of 20% trichloroacetic acid at 4°C. The medium was then washed with 10% and 5% trichloroacetic acid, extracted with ether, and dissolved in NaDodSO₄ sample buffer [125 mM Tris-HCl, pH 6.8/1% NaDodSO₄/5% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol]. NaDodSO₄/polyacrylamide gel electrophoresis was performed on 10% acrylamide slab gels with the discontinuous buffer system described by Maizel (13). Gel scanning was performed with a densitometer (E. C. Apparatus, St. Petersburg, FL).

RESULTS

When parathyroid gland slices or isolated cells were incubated for 120 min with ³²P_i and the medium proteins were precipitated and separated on polyacrylamide gel electrophoresis followed by radioautography, the dominant protein was a 68,000-dalton ³²P-labeled protein which eluted in a position corresponding to that of highly purified bovine PSP (Fig. 1). Gel filtration (Bio-Gel A-1.5m) of ³²P-labeled medium proteins revealed a peak of radioactivity in the position where marker ¹²⁵I-labeled PSP (¹²⁵I-PSP) eluted (Fig. 2). When both ³²P_i and [³⁵S]methionine were incubated with parathyroid slices for 4 hr and the medium was analyzed on the same column, [³⁵S]-methionine appeared in both the PSP and PTH peaks (5), whereas only PSP contained ³²P. The elution position of ¹²⁵I-PSP had previously been determined by its passage over the same column (5). When the column fractions on Fig. 2 were analyzed by radioimmunoassay for bovine PSP (5), immunoreactivity appeared only in the dominant peak at tube 45 which was phosphorylated. The higher molecular weight peak observed in Fig. 2 and on gel electrophoresis (Fig. 1) was not studied further.

When the peak of ³²P-labeled protein from the Bio-Gel column was analyzed by NaDodSO₄/polyacrylamide gel electro-

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Abbreviations: PSP, parathyroid secretory protein; PTH, parathyroid hormone.

FIG. 1. Phosphorylation of M_r 68,000 protein. Tissue slices or isolated bovine parathyroid cells (1.0×10^6) were incubated with $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$) in phosphate-free KRB buffer for 120 min. Trichloroacetic acid-precipitated proteins from the medium were subjected to electrophoresis on 10% acrylamide/0.26% bisacrylamide separating gel. The stacking gel was 3% acrylamide/0.35% bisacrylamide. After electrophoresis, the gels were stained, dried, and exposed to Kodak X-AR film. Lanes: 1, medium from incubation of slices; 2, medium from incubation of cells. M_r is shown $\times 10^{-3}$.

phoresis, a single 68,000-dalton band that comigrated with authentic purified bovine PSP was observed. When the phosphorylated protein was analyzed by isoelectrofocusing, the ^{32}P -labeled peak was identified at pH 4.6. When double-labeled protein (^{35}S methionine and ^{32}P) was analyzed, the two radio labels comigrated (Fig. 3).

The effect of calcium on the amount of radiolabeled M_r 68,000 protein secreted into the medium was also studied. Tissue slices were incubated in phosphate-free KRB buffer containing low (0.5 mM) and high (2.5 mM) concentrations of calcium. The amount of radioactivity (^{35}S and ^{32}P) in the M_r 68,000 protein was quantitated by Bio-Gel column chromatography and NaDodSO₄/polyacrylamide gel analysis. At 2.5 mM calcium, there was a significant reduction in both ^{32}P and [^{35}S]methionine recovered in the secreted protein (55–65% less than at 0.5 mM calcium). NaDodSO₄/polyacrylamide gel electro-

FIG. 2. Gel filtration of medium proteins on Bio-Gel A-1.5m. Parathyroid gland slices were incubated with $^{32}\text{P}_i$ (1 mCi) and [^{35}S]methionine (0.1 mCi) for 4 hr in phosphate-free KRB buffer. Chromatography of lyophilized medium was performed on a 1.2×75 cm column; 1.0-ml fractions were assayed for radioactivity.

FIG. 3. Isoelectric focusing of M_r 68,000 protein labeled with ^{32}P and [^{35}S]methionine. The peak of radioactively labeled protein (fraction 45 in Fig. 2) was analyzed on 4% polyacrylamide gels and on an isoelectric focusing gradient from pH 3.5 to 7.0.

phoresis of ^{32}P - and ^{35}S -labeled M_r 68,000 protein at 0.5 and 2.5 mM calcium is shown in Fig. 4.

The ratio of ^{32}P to ^{35}S in the M_r 68,000 protein in the medium at 0.5 mM Ca appeared to be similar to that at 2.5 mM Ca, which may indicate that the decrease in ^{32}P is primarily due to decreased secretion of newly synthesized PSP. Separate experiments have shown a 65–75% decrease in total secreted PSP at high Ca^{2+} (9).

Incubation of lysate prepared from isolated parathyroid cells with [γ - ^{32}P]ATP resulted in incorporation of ^{32}P into various proteins, but the most heavily labeled one observed was at 68,000 daltons (Fig. 5). The intensity of the M_r 68,000 band decreased with increasing calcium concentration from 0 to 3.0 mM. In separate incubations, the concentration of calcium was

FIG. 4. Effect of calcium on the secretion of double-labeled M_r 68,000 protein. Tissue slices were incubated for 4 hr at 37°C in phosphate-free KRB buffer containing 0.5 mM (○, Δ) or 2.5 mM (●, ▲) CaCl_2 , [^{35}S]methionine at 10 $\mu\text{Ci}/\text{ml}$, and $^{32}\text{P}_i$ at 100 $\mu\text{Ci}/\text{ml}$. The medium was analyzed by NaDodSO₄/polyacrylamide gel electrophoresis.

FIG. 5. Regulation of phosphorylation of M_r 68,000 protein by calcium. Isolated bovine parathyroid cells were washed with phosphate-free KRB (0.5 mM Ca^{2+}), and homogenized in 20 mM Hepes , $\text{pH } 7.0/10 \text{ mM MgCl}_2/0.1 \text{ mM dithiothreitol}$. The homogenate was incubated with $5 \text{ } \mu\text{M } [\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 Ci/mmol) in a final volume of 0.1 ml at 30°C for 2 min at different concentrations of calcium and EGTA. The reaction was terminated by addition of $50 \text{ } \mu\text{l}$ of sample buffer, and the samples were kept in a boiling water bath for 5 min . Polyacrylamide gel electrophoresis was performed on $50\text{-}\mu\text{l}$ aliquots of reaction mixture. Dried gel was exposed to X-Omat AR film. In lanes 1, 2, 3, and 4, the concentration of calcium was $0, 0.5, 1.0$, and 3.0 mM , respectively. In lanes 5, 6, 7, and 8, 2.0 mM EGTA was incubated with homogenate in the presence of $0, 0.5, 1.0$, and 3.0 mM calcium, respectively.

further decreased by adding 2.0 mM EGTA . In the absence of added calcium, the addition of 2 mM EGTA resulted in a significant increase in phosphorylation. Addition of increasing concentrations of calcium (calculated free Ca^{2+} concentrations at $0.5, 1.0$, and 3.0 mM calcium were $0.1 \text{ } \mu\text{M}, 0.28 \text{ } \mu\text{M}$, and 1.0 mM , respectively, on the basis of stability constant $K = 4.4 \times 10^6 \text{ M}^{-1}$) in the presence of EGTA resulted in a decrease in phosphorylation of PSP. The densitometric scans of the gels in

Fig. 5 are shown in Fig. 6. At increasing calcium concentrations in the absence of EGTA, the inhibition was $65\%, 77\%$, and 90% , respectively. In the presence of EGTA, the inhibition was $24\%, 29\%$, and 85% , respectively.

DISCUSSION

The present report provides evidence that PSP, a major protein secreted by the parathyroid gland, is a phosphoprotein. In these studies, we have chosen to analyze the medium for phosphorylated proteins rather than to examine the entire profile of phosphorylated cytoplasmic proteins in isolated cells. In the medium, PSP and PTH are the major secretory proteins, and we have been able to document that PSP is phosphorylated in the cell prior to its release. We have also isolated the phosphorylated protein by passage through Bio-Gel columns. The protein has a pI value of ≈ 4.6 , which is similar to that of highly purified homogeneous preparations of bovine PSP (6). A pI value in the range $4.9\text{--}5.2$ was reported for PSP made in *in vitro* translation systems as well as PSP derived from bovine tissue (14). The phosphorylated protein thus behaved identically to purified PSP on isoelectric focusing, column chromatography, and polyacrylamide gel electrophoresis.

It will be necessary to examine the kinetics of ^{32}P labeling of this protein over longer periods of time and to determine if an increase in the ratio of ^{32}P to ^{35}S methionine is achieved over time. We have routinely used 2- and 4-hr incubations because secretion of PSP increases linearly with time up to 4 hr (7–9), and regulation of secretion by calcium has been investigated at these time periods.

PSP is associated with PTH in the secretory granule (15, 16) and could play an important role in secretion of the hormone. Phosphorylation of soluble granule proteins and secretory gran-

FIG. 6. Densitometric scans of gels in Fig. 5. Dashed line marks the peaks representing labeled PSP.

ule membranes in the parathyroid cell may be important in exocytosis, but this has not been investigated in the parathyroid previously. Examples of secreted proteins that are phosphorylated include casein (17), ovalbumin (18), myosin light chain (19), and a M_r 68,000 protein from peritoneal mast cells (20). Phosphorylation of pro-ACTH/endorphin-derived peptides has also recently been described (21).

It is also important to study calcium and cAMP regulation of phosphorylation. In the *in vitro* system used, we have shown that phosphorylation of this protein is maximal in the presence of EGTA and in the absence of added calcium. It is tempting to speculate that the inhibitory effect of calcium on PTH secretion from parathyroid cells is mediated via phosphorylation/dephosphorylation mechanisms involving PSP. A possible mechanism for increased phosphorylation of this protein may include an increase in the local concentration of cAMP or cGMP brought about by inhibition of calmodulin-dependent phosphodiesterase activity through changes in cytosol calcium concentration (22, 23). Addition of EGTA to bovine parathyroid cell sonicates inhibits both cAMP and cGMP phosphodiesterase activities (24, 25).

Studies implicating calmodulin and calcium-dependent phosphorylation of endogenous proteins in several secretory systems have recently been reported (26–28). In all such instances, stimulation of protein phosphorylation was observed. Unlike other systems, however, PTH and PSP release is inhibited, not stimulated, by increased extracellular calcium concentration. Further studies involving intact cell systems will be needed to determine whether phosphorylation of PSP mediates the effects of calcium on PTH secretion (29).

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