Endocrine, *vol. 10, no. 1, 77–82, February 1999* 0969–711X/99/10:77–82/$11.50 © 1999 by Humana Press Inc. All rights of any nature whatsoever reserved.

GH Kinase Activity in Bovine Anterior Pituitary Subcellular Fractions

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Growth hormone (GH) and prolactin (PRL) share significant structural homology. We have previously characterized the phosphorylation of bovine PRL and wish to determine whether a similar kinase activity phosphorylates bovine GH. Phosphorylation of bovine GH was performed using [α-^{32}P]ATP labeling of subcellular fractions. Bovine GH phosphorylation was dependent on Zn^{2+} or Cu^{2+} with apparent Km's of 0.9 and 1.0 m*M*, respectively, and a pH maxima of 7.0. The apparent Km's of bovine GH kinase activity for exogenous bovine GH and ATP were 30 μ*M* and 376 μ*M*, respectively. Exogenous bovine PRL served as a competitive substrate, increasing the apparent Km for bovine GH by threefold compared to the Km determined without exogenous bovine PRL. We conclude: 1) in vitro phosphorylation of bovine GH occurs under conditions that are consistent with those found in anterior pituitary cells, and 2) a similar kinase activity phosphorylates both bovine PRL and GH.

Key Words: GH; kinase; phosphorylation; zinc; PRL; bovine.

Introduction

Growth hormone (GH) and prolactin (PRL) share many features. They are hormones belonging to the same protein family as placental lactogens *(1)*. The PRL and GH gene are believed to have arisen by gene duplication from a common ancestral protein *(2)* and both the gene *(3)* and the expressed protein *(4)* share significant sequence homology. The three dimensional structures for human *(5)* and rabbit *(6)* PRLs are predicted to be similar to that of GH (*7,8*, Protein Data Base # 3HHU) based on sequence homology alignment and homolog modeling studies. Both PRL and GH initiate their cellular actions by binding to receptors belonging to the same subgroup of the cytokine receptor family *(9,10)* that activate members of the JAK and STAT protein families to promote their effects within the cell *(11)*. PRLs and GHs have evolved independent biological actions *(12,13)*, although primate GHs still possess substantial lactogenic activity. The pituitary cells which produce GH and PRL are also closely related. Discrete PRL and GH producing cells are derived from a population of acidophilic staining somatomammotrophic cells in the pituitary that synthesize both protein hormones.

During synthesis, PRL and GH both undergo a variety of posttranslational modifications, many of which are known to occur in the Golgi of the cells of the pituitary, e.g., glycosylation and sulfation *(14)*. Phosphorylation of GH and PRL have been reported in vivo and in vitro in several species *(15–17)*. bPRL is phosphorylated in vivo at serines 26, 34, and 90 *(18)* with stoichiometric ratios of 1:1:10. Phosphorylation of bPRL reduces its biological activity *(19)* by reducing its ability to bind the PRL receptor *(20)*. Using glutamic acid mimicry of phosphorylation at each of the three phosphorylation sites of bPRL, serine 90 was shown to be responsible for the inactivation of bPRL by phosphorylation *(21)*. Sequence comparisons of bPRL and bGH show that the phosphorylation sites at serine 90 of bPRL is conserved at residue 85 of bGH, while bPRL phosphorylation sites at serines 26 and 34 are not conserved in bGH.

PRL is phosphorylated in secretory granules *(16,22,23)* and, although not to the same degree, by other cellular organelles along the secretory pathway *(23)*. When phosphorylated bPRL is isolated under conditions that minimize phosphatase activity *(24)* most bPRL is found in a phosphorylated form (Brooks, unpublished). The optimal conditions for phosphorylation of bPRL have been determined in vitro and were found to be consistent with a physiologically relevant process *(23)*. In contrast, neither the cellular location nor optimal conditions for GH phosphorylation have been determined. We hypothesize that GH phosphorylation may share many properties of phosphorylation previously described for PRL.

The purpose of this study was to define the parameters of bGH phosphorylation and compare these findings to those of bPRL phosphorylation. We show phosphorylation of bGH occurs under conditions that are similar, although

Received October 26, 1998; Revised December 21, 1998; Accepted January 7, 1999.

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not identical, to those of bPRL. Furthermore, substrate competition studies demonstrate a kinase activity is present in membranous fractions of the bovine pituitary that is able to phosphorylate both bPRL and bGH.

Results

Phosphorylation of bGH by Subcellular Fractions of Bovine Anterior Pituitary

The technique used for subcellular fractionation has been characterized previously *(23,25)*. Briefly, cellular fractions have been characterized by SDS-containing gel electrophoresis and transmission electron microscopy. Microscopy showed fraction 5 was composed of dense secretory granules with a surrounding, but incomplete, membrane. Fraction 5 was depleted of free membranes. Electrophoretic analysis of the proteins in fraction 5 showed large amounts of endogenous bGH and bPRL and small amounts of other proteins. Fraction 4 consisted primarily of membranes but also contained several dense secretory granules. The endogenous bGH and bPRL represented a small proportion of the protein when compared to fraction 5. Other fractions were devoid of secretory granules and contained very low or undetectable amounts either bGH or bPRL.

Phosphorylation of endogenous bGH was present in all fractions with the highest activity present in Fraction 5 which is enriched for secretory granules and contained the greatest amount of endogenous bGH (data not shown). Phosphorylation of bGH in fractions 1 and 2 were the lowest and were not further characterized. Reactions were run with and without 30 μg (13.7 μ*M*) of exogenous National Hormone and Pituitary Program (NHPP) bGH added in order to account for the presence of unequal amounts of endogenous bGH in membrane fractions. Increased bGH phosphorylation was observed in all fractions with the addition of exogenous NHPP-bGH. The absolute increase in bGH phosphorylation was greatest in fraction 5, but when expressed as a percentage (Fig. 1) increases in bGH phosphorylation were not statistically different between fractions.

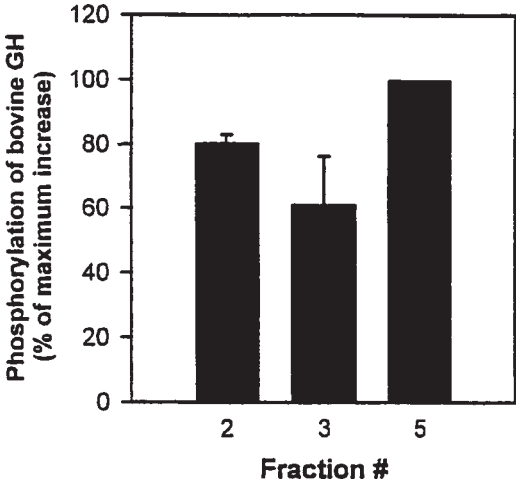

Fig. 1. Phosphorylation of exogenous bGH by bovine anterior pituitary subcellular fractions 2, 3. and 5. Subcellular fractions 2, 3, and 5 (25 μg) were incubated for 10 minutes with 50 μCi of [γ-^{32}P]ATP in 50 m*M* MOPS pH 7.0, 0.5 m*M* EGTA, 1 m*M* ZnSO4, 50 m*M* NaF, 200 μ*M* ATP with or without 13.6 μ*M* NHPP bGH in 100 μL reaction volume. ^{32}P-labeled bGH was separated by SDS-containing polyacrylamide gel electrophoresis, excised and the associated radioactivity measured. The difference in values with and without exogenous bGH were determined and expressed as a percent of the values obtained for fraction 5. Values represent the mean ± SEM for three pituitary preparations.

Degree of Phosphorylation as a Function of Cation and pH

Characterization of optimal cation concentrations for kinase activity were determined by titration experiments using fraction 5 (data not shown). Various concentrations of Ca^{2+}, Mg^{2+}, or Mn^{2+} failed to increase bGH phosphorylation over that of basal levels (no cation present) (Fig. 2). The presence of Cu^{2+} (1.8 m*M*) and Zn^{2+} (1.8 m*M*) increased phosphorylation above the basal rate by 44 and 55% respectively (Fig. 2). The effects on phosphorylation by Zn^{2+} and Cu^{2+} were dose-dependent (Fig. 3); apparent Km's for Zn^{2+} and Cu^{2+} in the phosphorylation of bGH were 0.9 ± 0.4 m*M* and 1.0 ± 1.4 m*M*, respectively.

Phosphorylation of bGH was evaluated over a pH range of 5.9 to 8.3. ^{32}P-phosphate incorporation into bGH was maximum within the pH range of approx 6.6 to 7.3, with phosphorylation dropping off at pH's which were either lower or higher (Fig. 4).

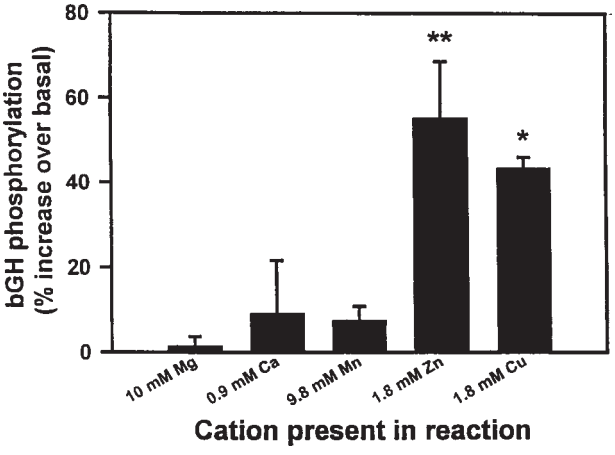

Fig. 2. Phosphorylation of exogenous bGH by bovine anterior pituitary subcellular fraction 5 as a function of free cation. Subcellular fraction 5 (25 μg) was incubated with [γ-^{32}P]ATP (50 μCi) in 50 m*M* MOPS pH 7.6, 0.5 m*M* EGTA, 50 m*M* NaF, 200 μ*M* ATP, and 13.6 μ*M* NHPP bGH (30 μg) in 100 μL reaction volumes with either 1 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 1 m*M* $CuSO_4$, 10 m*M* $MnCl_2$, or 1 m*M* $ZnSO_4$ for 10 min. The amount of bGH phosphorylation was measured as a percent increase over GH phosphorylation with no cations present (basal). Values represent the mean ± SEM for three pituitary preparations. Differences between groups were compared by a one-way analysis of variance followed by Tukey's multiple comparison test (*, $p < 0.05$, and **, $p < 0.01$ when compared to phosphorylation in the absence of added cations).

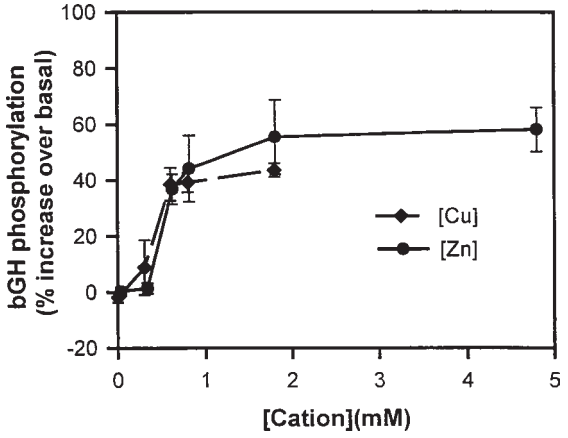

Fig. 3. Phosphorylation of exogenous bGH by bovine anterior pituitary subcellular fraction 5 as a function of free Cu^{2+} or Zn^{2+} concentration. Subcellular fraction 5 (25 μg) was incubated with [γ-^{32}P]ATP (50 μCi) in 50 m*M* MOPS pH 7.6, 0.5 m*M* EGTA, 50 mM NaF, 200 μ*M* ATP, and 13.6 μ*M* NHPP bGH in 100 μL reaction volume with variable concentrations of ZnSO4 (up to 10 m*M*) or $CuSO_4$ (up to 2 m*M*) for 10 min. The amount of bGH phosphorylation was measured as a percent increase over GH phosphorylation with no additional cation present (basal). Values represent the mean ± SEM for three pituitary preparations.

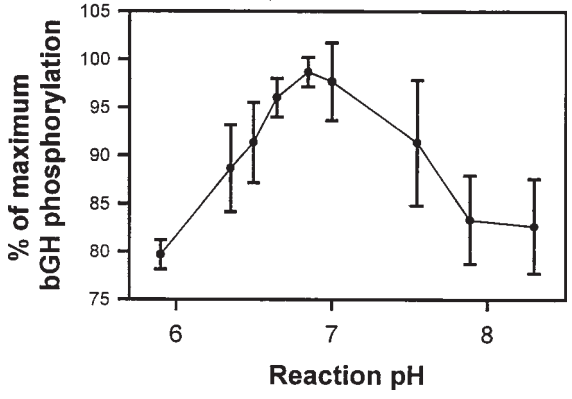

Fig. 4. Phosphorylation of exogenous bGH by bovine anterior pituitary subcellular fraction 5 as a function of reaction pH. Subcellular fraction 5 (25 μg) was incubated with [γ^{32}P]ATP (50 μCi) in 50 m*M* MOPS, 0.5 mM EGTA, 1 m*M* $ZnSO_4$, 50 m*M* NaF, 200 μ*M* ATP, and 13.6 μ*M* NHPP bGH in 100 μL reaction volume for 10 min at the pH indicated for each point. The amount of bGH phosphorylation was measured as a percent of the phosphorylation measured at pH 7.0. Values represent the mean ± SEM for three pituitary preparations.

Apparent Km's of bGH Kinase Activity for Exogenous bGH or ATP

Phosphorylation of bGH was evaluated over increasing doses of exogenous NHPP-bGH up to 15.9 μ*M* in the presence of 1.8 m*M* Zn^{2+}, and 200 μ*M* ATP, at pH 7.0 for 10 min. The apparent Km of the kinase activity for bGH was 30 μ*M*. Similarly, the apparent Km of the kinase for ATP was 376 μ*M* in the presence of 1.8 m*M* Zn^{2+}, at pH 7.0 for 10 min and ATP concentrations ranging from 20–300 μ*M* (Fig. 5).

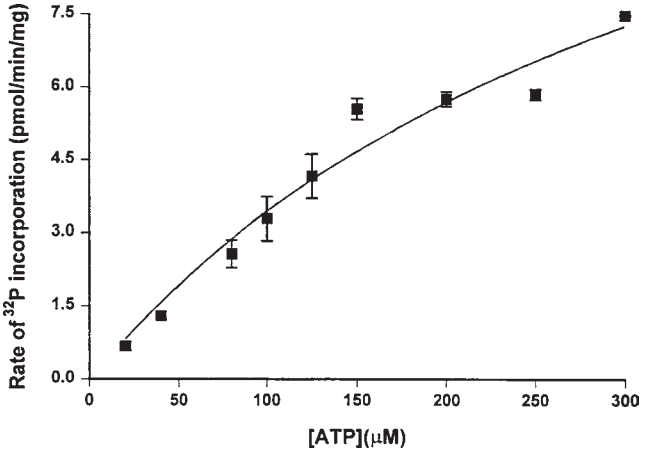

Fig. 5. Rate of incorporation of ^{32}P into exogenous bGH by bovine anterior pituitary subcellular fraction 5 as a function of ATP concentration. Subcellular fraction 5 (25 μg) was incubated with [γ-^{32}P]ATP (50 μCi) in 50 m*M* MOPS, pH 7.0, 0.5 m*M* EGTA, 1 m*M* $ZnSO_4$, 50 m*M* NaF, and 13.6 μ*M* NHPP bGH in the presence of variable concentrations of ATP in 100 μL reaction volume for 10 min. Curve fitting to hyperbolic Michaelis-Menton kinetics was performed using FigP software. Points represent the mean of two pituitary preparations.

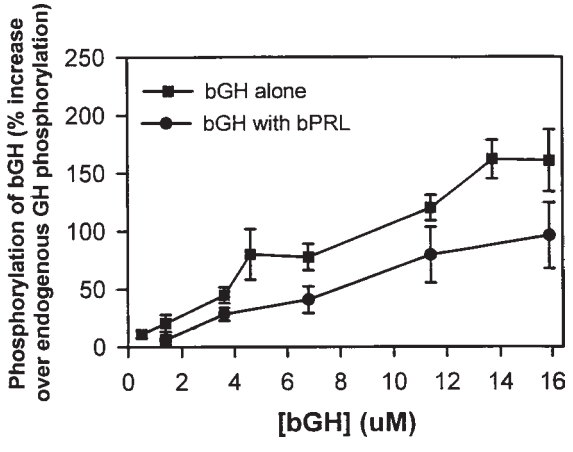

Fig. 6. Phosphorylation of exogenous bGH by bovine anterior pituitary subcellular fraction 5 with or without exogenous bPRL. Subcellular fraction 5 (25 μg) was incubated with [γ-^{32}P]ATP (50 μCi) in 50 m*M* MOPS, pH 7.6, 0.5 m*M* EGTA, 1 m*M* $ZnSO_4$, 50 m*M* NaF, 200 μ*M* ATP, variable concentrations of NHPP bGH, and either with or without exogenous bPRL (8.3 μ_M) in 100 μL reaction volume for 10 min. The amount of bGH phosphorylation was measured as a percent increase over PRL phosphorylation with no exogenous GH added (endogenous GH phosphorylation). Values represent the mean ± SEM for three pituitary preparations.

Apparent Km of bGH Kinase Activity for Exogenous bGH in the Presence of Exogenous bPRL

To assess whether bGH and bPRL are being phosphorylated by the same kinase activity, experiments were designed to determine if exogenous bPRL functioned as a competitive substrate in bGH kinase assays. bGH phosphorylation was measured in the presence of increasing

amounts of NHPP bGH in either the absence or presence of NHPP-bPRL (8.3 μ*M*) (Fig. 6). The apparent Km for bGH was increased from 30–95 μ*M* by the presence of 8.3 μ*M* bPRL. The calculated Vmax was not influenced by the addition of exogenous bPRL. The reduction in bGH phosphorylation resulting from the addition of exogenous bPRL was analyzed by a two-way ANOVA (treatment and experiment) and found to be significant at a level of $p < 0.05$.

Discussion

We have previously shown that both endogenous and exogenous bPRL is phosphorylated in multiple subcellular fractions of bovine anterior pituitaries, most significantly in fractions enriched for secretory granules *(23)*. In this report we show that endogenous and exogenous bGH is phosphorylated under similar conditions to those of bPRL. Several pieces of evidence support the hypothesis that the same kinase phosphorylates both bGH and bPRL.

The structures of bGH and bPRL are similar: bPRL and bGH share significant amino acid sequence homology *(4)*. The three-dimensional structure of GH has been described for porcine *(7)* and human *(8)* GHs and the three dimensional structures of human *(5)* and rabbit *(6)* PRLs have been prepared by homology modeling techniques. These studies predict tertiary structures similar to thoe of GH. Phosphorylated bPRL has been isolated and characterized from bovine anterior pituitaries *(16,18,24)*, and a major site of phosphorylation was identified at serine 90 with minor sites at serines 26 and 34. The residue corresponding to serine 90 is conserved in PRL's and GH's from a number of species *(4)*, and therefore could serve as a potential site of phosphorylation in GH, particularly in light of the predicted structural similarities. In rat PRL, serine 177 has been identified as the primary site of in vitro phosphorylation with minor phosphorylations at either threonine 58 or 63 *(26)*. Neither threonine 63 nor serine 177 are conserved in bGH, ruling these residues out as GH phosphorylation sites. However, the residue corresponding to threonine 58 is present as a serine in the GHs and therefore could be phosphorylated by a serine/threonine kinase.

We have characterized the optimal conditions for bGH phosphorylation in this in vitro system. The pH range for both bPRL and bGH phosphorylation overlap considerably. A significant similarity to our previous findings for bPRL is that phosphorylation of both bPRL and bGH is enhanced in the presence of zinc. The apparent Km for Zn^{2+} is 0.6 m*M* for bPRL *(23)*, similar to the 0.9 m*M* determined for bGH. Both PRL *(26,28,29)* and GH *(30,31)* are Zn^{2+} binding proteins. Whether Zn^{2+}-dependant phosphorylation of bGH is associated with zinc binding to the hormone, kinase or other components present in the reaction is uncertain. Phosphorylation is not affected by Mg^{2+}or Ca^{2+}, two cations that are known to be frequently involved in kinase reactions. On the other hand, Mn^{2+} enhanced bPRL phosphorylation while having little effect on bGH phosphorylation, and Cu^{2+}, which significantly enhances bGH phosphorylation, has a much more subtle effect on bPRL phosphorylation. Such subtle differences in the metal-dependance of phosphorylation and the known metal-binding properties of these proteins suggests that the metal binds to the hormone and modulates substrate availability most likely by inducing a specific protein conformation that influences the Km for the reaction. We have previously demonstrated that zinc-binding to bPRL induces subtle structural changes *(28)*. Zinc is found in high concentrations in the pituitary *(32)*, thus the binding of zinc to these proteins may regulate their phosphorylation in these cells.

The apparent Km's for ATP for kinase-mediated phosphorylation of bPRL and bGH are 267 and 376 μ*M*, respectively. These ATP concentrations are similar to physiologic concentration found within the cell.

The most convincing evidence that bPRL and bGH are phosphorylated by the same kinase activity is the reduction in bGH phosphorylation in the presence of modest concentrations of exogenous bPRL. The presence of a sub-maximal dose of NHPP-bPRL (8.3 μ*M*) effectively increased the apparent Km of bGH from 30 to 95 μ*M*. A modest concentration of a competitive substrate, i.e., one that binds to the same active site on the kinase, should cause a reduction in the reaction rate which will be reflected by a reduction in the apparent Km, with no significant change in Vmax, similar to what was seen here. The apparent affinities of these substrates for the kinase were similar: the apparent Km for bPRL is 15 μ*M* *(23)*, whereas that for bGH is 30 μ*M*.

Based on the findings, we conclude that bGH can be phosphorylated by secretory granules of the bovine anterior pituitary, and to a lesser extent by other subcellular membrane fractions when sufficient exogenous bGH substrate is provided. The optimal conditions under which phosphorylation occurs in this in vitro system are markedly similar to those of bPRL *(23)*. Further, bPRL displays the kinetic characteristics of a competitive substrate in bGH kinase reactions, suggesting that the same kinase is responsible for phosphorylation of both bPRL and bGH.

Materials and Methods

Subcellular Fractionation

Subcellular fractionation was performed at 4°C according to methods previously described *(23)* with minor modifications. Bovine anterior pituitaries obtained fresh from a local meat packer were homogenized in 3.3 volumes of 0.05 *M* Tris-HCl, pH 7.4, 0.3 *M* sucrose (buffer A) with protease inhibitors (100 μ*M* leupeptin, 1 μ*M* pepstatin A, 100 μ*M* TPCK, and 5 m*M* EDTA), using a motor-driven Teflon-glass homogenizer (8–10 strokes at 800 rpm). The homogenate was centrifuged at 3000*g* for 10 min. The pellet was suspended at half the volume and re-centrifuged as

above. The supernatants were combined and centrifuged at 45,000*g* for 20 min. The pellet was suspended in a small amount of buffer A, adjusted to a density of 1.18 g/mL using 70% sucrose, and inserted into a discontinuous sucrose density gradient containing components with the following densities: 1.14, 1.16, 1.18, 1.20, and 1.22 g/mL. The sucrose gradient was centrifuged at 96,000*g* for 2 h. Membrane fractions were collected at each interface (fractions 1–4 respectively), and the pellet (fraction 5). Each fraction was diluted with buffer A and centrifuged at 150,000*g* for 30 min. The pellets were suspended in buffer A and stored at –80°C. The protein concentration of each fraction was measured by the method of Lowry *(33)*.

This technique produces fractions highly enriched for plasma membrane (fraction 1), endoplasmic reticulum and Golgi (fractions 2 and 3), mitochondria and secretory granules (fraction 4) and secretory granules (fraction 5) *(25)*. Our characterization of these fractions *(23)* are consistant with the report of Lorenson and Jacobs *(25)*. Each Experiment was performed at least 3 times on separate preparations of subcellular fractions, therefore, the reported measures of variance include those generated by heterogeneity in the preparation of subcellular fractions.

Protein Kinase Assay

Subcellular fractions were incubated in 50 m*M* MOPS, pH 7.0, 0.5 m*M* EGTA, 1 m*M* ZnSO4, 50 m*M* NaF, 200 μ*M* ATP and 50 μCi [γ-^{32}P]ATP (7000 Ci/mMol, ICN Pharmaceuticals Inc., Costa Mesa, CA) in a 100 μL reaction volume at 30°C for 10 min unless otherwise indicated. bGH and bPRL (biological grade) were provided by the NHPP and were utilized as sources of exogenous hormone. Free ion con-centrations in kinase reactions were determined by multiple equilibria calculations (Eqcal software, Biosoft, Cambridge, United Kingdom) and are presented in parentheses in the text and in the axes of figures. Kinase reactions were terminated by the addition of 50 μL of a solution containing 20% glycerol, 9% SDS, 10% β-mercaptoethanol, 125 m*M* Tris-HCl, pH 6.8, 3 m*M* EDTA, and 0.02% Pyronin Y, followed by a 2-min treatment in boiling water. Proteins, including bGH and bPRL, were separated by SDS-containing 12% polyacrylamide gel electrophoresis performed under reducing conditions *(34)* followed by staining using Coomassie Brilliant Blue-R. Measurement of ^{32}P-phosphate associated with bGH was performed by excision of the protein band co-migrating with NHPP-bGH and measurement of the associated radioactivity by liquid scintillation spectrophotometry. Alternatively, gels were dried and the relative intensity of bGH-associated β-radiation evaluated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Apparent Km's for the kinase activities were calculated from data fit to hyperbolic Michaelis-Menton kinetics (FigP software, Biosoft, Cambridge, United Kingdom).

Acknowledgments

Supported by the National institutes of Health (DK42604 and DK01989 to C. L. B.) and a Physician Scientist Award (DK021209 to J. R. W.). Hormones were kindly provided by National Hormone and Pituitary Program.

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