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Inactivation of Bovine Kidney Cytosolic Protamine Kinase by the Catalytic Subunit of Protein Phosphatase 2A

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Incubation of highly purified preparations of the bovine kidney cytosolic protamine kinase in the presence of near homogeneous preparations of the catalytic subunit of protein phosphatase 2A ($PrP2A_c$) from bovine kidney resulted in time-dependent inactivation of the protamine kinase. By contrast, incubation of bovine kidney cytosolic casein kinase II with $PrP2A_c$ had no effect on the activity of this casein kinase II. In the presence of 10 mM sodium fluoride, 10 mM inorganic orthophosphate, 1 mM pyrophosphate or 0.1 mM ATP, the inactivation of the protamine kinase by $PrP2A_c$ was completely inhibited. Half-maximal inhibition by ATP occurred at about 20 μM. The rate of inactivation of the protamine kinase by $PrP2A_c$ was unaffected by Mg^{2+}, Mn^{2+}, Ca^{2+}, EDTA or EGTA at 1 mM. The results strongly indicate that the activity of the cytosolic protamine kinase is regulated by phosphorylation/dephosphorylation.

The bovine kidney cytosolic protamine kinase is composed of a single polypeptide of apparent M_r ~ 45,000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel permeation chromatography (1). This enzyme was differentiated from other protein kinases by its unique substrate specificity and chromatographic properties (1,2). The physiological role of the bovine kidney cytosolic protamine kinase has not yet been elucidated, and to date, physiologically relevant regulators of this enzyme had not been identified. However, the activity of a protamine kinase that exhibits catalytic and chromatographic properties identical to the bovine kidney enzyme was stimulated rapidly following treatment of isolated rat hepatocytes with insulin indicating a role for this enzyme in cellular responses to this hormone (2).

The abbreviations used are: PrP2A, protein phosphatase 2A; $PrP2A_c$, protein phosphatase 2A catalytic subunit; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N' tetraacetic acid.

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In this communication, we report that the highly purified protamine kinase of bovine kidney is inactivated following incubation with near homogeneous preparations of the catalytic subunit of protein phosphatase 2A ($PrP2A_c$). The results strongly indicate that the activity of the bovine kidney protamine kinase is regulated by phosphorylation/ dephosphorylation. The possible relevance of the results to the mechanism by which the activity of the protamine kinase was stimulated by insulin in isolated rat hepatocytes is discussed.

Materials and Methods

Affigel Blue gel was obtained from Biorad. Phosphorylase b, phosphorylase kinase and casein were obtained from Sigma Chemical Company. All other materials are given in references 1 and 2.

Purification of the bovine kidney cytosolic protamine kinase to apparent homogeneity (1) and partial purification of the rat hepatocyte protamine kinase (2) were as described. Determination of protamine kinase activity was as documented (2). One unit of protamine kinase activity was the amount of enzyme which catalyzed the incorporation of 1 nmol of phosphoryl groups into protamine. Protein was determined by the procedure of Bradford (3). Polyacrylamide gel electrophoresis was performed in slab gels (12% acrylamide) with 0.1% sodium dodecyl sulfate and Tris/glycine buffer, pH 8.3 (4). Protein bands were detected by staining with Coomassie Blue or silver (5).

^{32}P-labeled phosphorylase a was prepared from phosphorylase b using phosphorylase kinase, and phosphorylase phosphatase activity was determined as described (6,7). One unit of phosphorylase phosphatase activity was the amount of enzyme which catalyzed the release of 1 nmol inorganic phosphate/min. The catalytic subunit of PrP2A was purified to apparent homogeneity from bovine kidney cytosol by a procedure similar to the one employed to purify $PrP2A_c$ from rabbit skeletal muscle and liver (8,9). The final preparations consisted of a single Coomassie Blue or silver staining polypeptide of apparent M_r ~ 36,000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

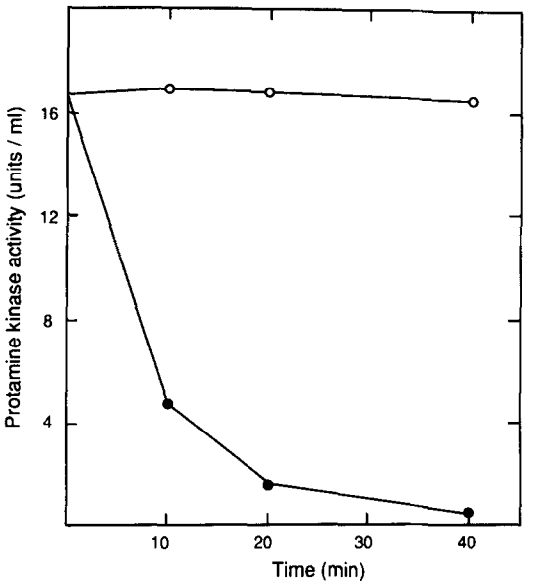
The criteria employed to identify the purified phosphatase preparations as type 2A (10,11) were that the enzyme specifically dephosphorylated the α subunit of phosphorylase kinase, that the enzyme was divalent cation-independent and that ATP at 0.1 mM completely inhibited its activity with ^{32}P-labeled phosphorylase. Half-maximal inhibition with ATP occurred at about 20 μM ATP (see Fig. 3 below). ^{32}P-labeled phosphorylase kinase was prepared by incubation with the catalytic subunit of cAMP-dependent protein kinase as described (11,12). Dephosphorylation of the α subunit by $PrP2A_c$ was analyzed by autoradiography following resolution of ^{32}P-labeled phosphorylase kinase α and β subunits by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Casein kinase II was purified to apparent homogeneity from bovine kidney cytosol by a procedure similar to the one employed to purify the mitochondrial casein kinase II except that chromatography of the preparations on heparin-agarose was omitted (13). These casein kinase II preparations consisted of two Coomassie Blue or silver staining subunits of apparent M_r ~ 36,000 and 28,000 as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Casein kinase II activity was determined as described (13) except that Mg^{2+} at 10 mM instead of 1.5 mM

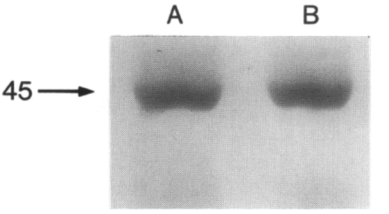
was employed in the incubations. One unit of casein kinase II activity was the amount of the enzyme which catalyzed the incorporation of 1 nmol of phosphoryl groups into casein (13).

Results and Discussion

Incubation of near homogeneous preparations of the bovine kidney cytosolic protamine kinase with $PrP2A_c$ resulted in time-dependent inactivation of the protamine kinase preparations (Fig. 1). By contrast, the activity of purified preparations of the bovine kidney casein kinase II were unaffected by incubation with any of the $PrP2A_c$ preparations employed (not shown). The inactivation of the kidney protamine kinase by $PrP2A_c$ was not due to contaminating protease(s), if any, because the



(1)



(2)

Fig. 1. Inactivation of bovine kidney cytosolic protamine kinase by $PrP2A_c$ - Highly purified protamine kinase (170 units/ml) was incubated at 30°C in the absence (o---o) and presence (•---•) of $PrP2A_c$ (7 unit/ml) in 50 mM imidazole chloride, pH 7.3, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.1 mM EGTA, and 14 mM β-mercaptoethanol in a final volume of 0.1 ml. At the indicated times, a 0.01 ml aliquot of the incubations was used to determine protamine kinase activity as described (1) except that 3 min instead of 10 min assays were performed.

Fig. 2. Incubation of the protamine kinase in the absence (A) and presence (B) of $PrP2A_c$ - The conditions were as described in the legend to Fig. 1 except that following incubation of the protamine kinase with $PrP2A_c$ for 1 h, 50 µl of sample buffer was added (7). The solutions were then heated at 100°C for 5 min and sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out (7). The gel, 12% acrylamide was stained with silver (5). The arrow denotes the position of the protamine kinase.

apparent M_r (~ 45,000) of the kidney kinase as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was not altered following incubation with $PrP2A_c$ (Fig. 2). In addition, the inactivation of the protamine kinase by $PrP2A_c$ was completely inhibited in the presence of the general protein phosphatase inhibitors sodium fluoride at 5 mM, pyrophosphate at 1 mM, or inorganic orthophosphate at 10 mM (not shown). ATP, a specific PrP2A inhibitor at 0.1 mM (11,12), completely inhibited the inactivation of the protamine kinase by $PrP2A_c$. Half-maximal inhibition occurred at about 20 μM ATP (Fig. 3). This is similar to the concentration of ATP required to observe half-maximal inhibition of $PrP2A_c$ with phosphorylase a (Fig. 3 and Refs. 11,12) and with other substrates of $PrP2A_c$ (11,12).

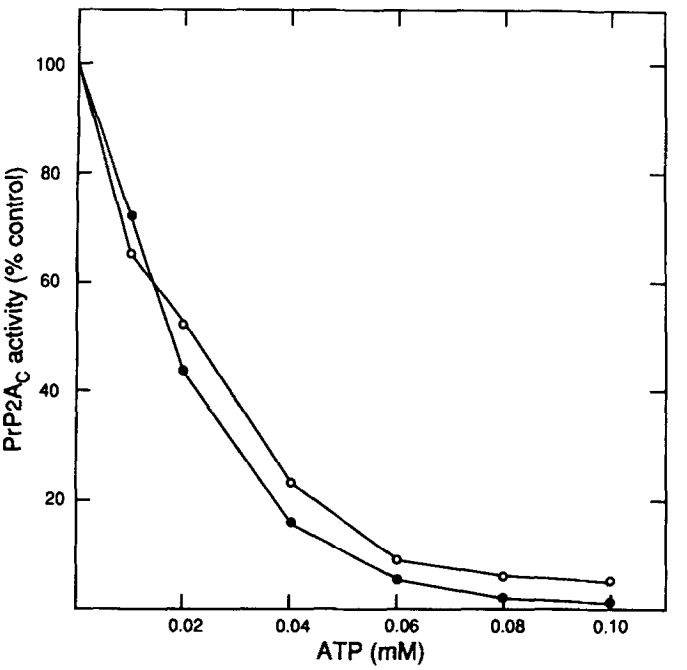


Fig. 3. Inhibition of $PrP2A_c$ by ATP - Incubation of the protamine kinase with $PrP2A_c$ for 10 min in the presence of ATP and assay of the protamine kinase for 3 min were performed (•---•) as described in the legend to Fig. 1. The 100% control value refers to the maximal inactivation of the protamine kinase observed in the absence of ATP. The effect of ATP on the phosphorylase phosphatase activity of $PrP2A_c$ (o---o) was determined as described (11). The 100% control value for phosphorylase phosphatase was the activity determined in the absence of ATP.

The rate of protamine kinase inactivation by the $PrP2A_c$ preparations was unaffected by 1 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, EDTA, EGTA or sodium vanadate. The inactivation of the cytosolic protamine kinases by the purified $PrP2A_c$ was completely inhibited by 200 mM NaCl. Half-maximal inhibition by NaCl occurred at about 50 mM (Fig. 4).

The results presented in this report strongly indicate that the activity of the protamine kinase isolated from kidney cytosol is inactivated by dephosphorylation, and therefore that the purified kidney enzyme may contain covalently bound regulatory phosphoryl groups. This protamine kinase phosphorylation could have occurred either during the course of the purification and/or in vivo. The latter possibility appears to be the most likely because the activity of the protamine kinase determined in kidney extracts does not increase during the purification of this enzyme (1). In addition, all the buffers employed in the purification contain 1 mM EDTA (1). Therefore, during the isolation of the protamine

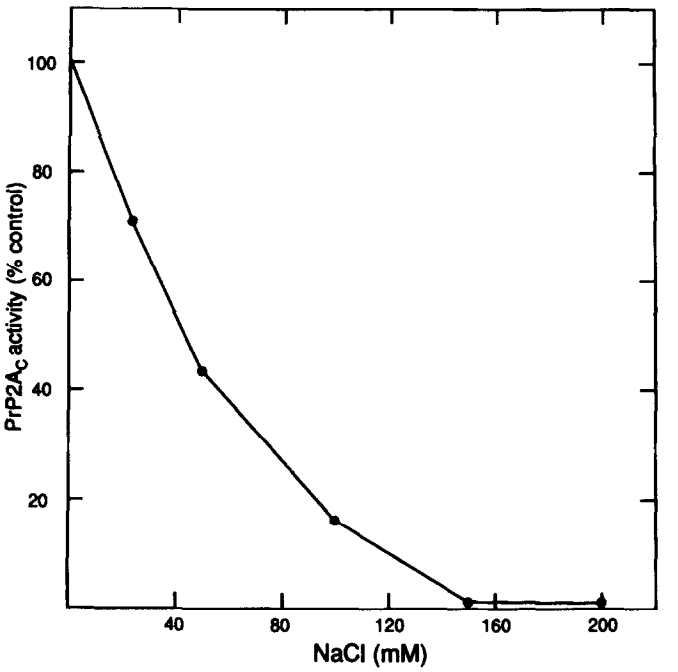


Fig. 4. Inhibition of $PrP2A_c$ by NaCl - Incubation of the protamine kinase with $PrP2A_c$ for 10 min in the absence and presence of NaCl, and assay of the protamine kinase for 3 min were as described in the legend to Fig. 1.

kinase, divalent-cation dependent phosphorylation of this enzyme can be expected to be inhibited. It is also pertinent to note that homogenization is performed in a phosphate buffer (1). Therefore, at the first steps of the purification, protamine kinase protein phosphatase(s) is(are) likely to be completely inhibited. In addition, at all the later stages of the purification the protamine kinase is present in solutions containing NaCl at a concentration > 200 mM. Under these conditions, the $PrP2A_c$ catalyzed inactivation of the protamine kinase is completely inhibited (Fig. 4). Therefore, dephosphorylation catalyzed by PrP2A cannot account for any loss of activity during the purification. Whether protein phosphatases other than PrP2A regulate the activity of the protamine kinase, remains to be determined.

Protein phosphatase 2A is relatively specific for phosphoseryl and phosphothreonyl residues. With some proteins, the enzyme has been reported to exhibit low but detectable activity towards phosphotyrosine residues (14,15). Therefore, it is conceivable that $PrP2A_c$ inactivates the protamine kinase preparations by dephosphorylation of phosphotyrosyl residues. However, based on preliminary analysis of the phosphoamino acid composition of the cytosolic protamine kinase which indicated the presence of phosphoseryl and not phosphothreonine nor phosphotyrosyl residues, it seems almost certain that $PrP2A_c$ catalyzes the dephosphorylation of phosphoserine residues present on the purified protamine kinase.

It is possible that a covalent phosphorylation of the protamine kinase may be involved in the mechanism by which the activity of this enzyme was stimulated in response to insulin in isolated rat hepatocytes. This idea was suggested previously (2) based on the following observations. First, it was necessary to include sodium fluoride, a general protein phosphatase inhibitor in the homogenization buffers to detect the effect of insulin on the rat hepatocyte protamine kinase. Second, the insulin-stimulated increase in protamine kinase activity was stable to chromatography on protamine-agarose, DEAE-cellulose, and Sephacryl S-200

gel filtration indicating that a covalent modification of the kinase was involved. Third, the apparent M_r of the kinase from insulin-treated and control cells was about 45,000 as estimated by gel permeation chromatography on Sephacryl S-200 indicating that insulin had not stimulated kinase activity by limited proteolysis or via modulation of the association of the kinase with a regulatory factor. Fourth, cyclohexiimide, a protein synthesis inhibitor, did not prevent the insulin-stimulated increase in protamine kinase activity indicating that insulin had stimulated the activity of a pre-existing pool of the kinase. Clearly, the possibility that insulin-stimulation of the hepatocyte protamine kinase may occur via increased phosphorylation of the protamine kinase is supported by the results presented in this communication. Moreover, experiments utilizing partially purified preparations of the hepatocyte protamine kinase were also inactivated following incubation with $PrP2A_c$ (not shown). In this regard, it is interesting to note that purified preparations of an insulin-stimulated ribosomal protein S6 kinase (16) and partially purified preparations of an insulin-stimulated microtubule-associated protein-2 kinase (16) have also been shown to be inactivated following incubation with $PrP2A_c$. In addition, phosphorylation and partial reactivation (about 30% of maximum) of the ribosomal protein S6 kinase by the microtubule-associated protein-2 kinase preparations has been reported (16). The kinase(s) which would catalyze the phosphorylation and concomitant reactivation of the $PrP2A_c$ inactivated protamine kinase has not yet been identified. It is possible, however, that together with the ribosomal protein S6 kinase and the microtubule-associated protein-2 kinase, the protamine kinase may participate in an insulin-stimulated cascade of sequential protein kinase phosphorylation/activation reactions.

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References

1. Damuni, Z., Amick, G.D., and Sneed, T.R. (1989) J. Biol. Chem. 264, 6412-6416.
2. Reddy, S.A.G., Amick, G.D., Cooper, R.H., and Damuni, Z. (1989) J. Biol. Chem. in press.
3. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
4. Laemmli, U.K. (1970) Nature 227, 680-685.
5. Merril, C.R., Goldman, D., Sedman, S.A., and Ebert, M.M. (1981) Science 211, 1437-1438.
6. Tung, H.Y.L., Pelech, S., Fisher, M.J., Pogson, M.J., and Cohen, P. (1985) Eur. J. Biochem. 149, 305-313.
7. Stewart, A.A., Hemmings, B.A., Cohen, P., Goris, J., and Merlevede, W. (1981) Eur. J. Biochem. 115, 197-205.
8. Tung, H.Y.L., Resink, T.J., Hemmings, B.A., Shenolikar, S., and Cohen, P. (1984) Eur. J. Biochem. 145, 51-56.
9. Lee, E.Y.C., Silberman, S.R., Ganapathi, M.R., Paris, H., and Petrovic, S. (1981) Cold Spring Harb. Conf. Cell. Prolif. 8, 425-439.
10. Ingebritsen, T.S., and Cohen, P. (1983) Science 221, 331-338.
11. Ingebritsen, T.S., Foulkes, J.G., and Cohen, P. (1980) FEBS Lett. 119, 9-15.
12. Ingebritsen, T.S., Foulkes, J.G., and Cohen, P. (1983) Eur. J. Biochem. 132, 263-274.
13. Damuni, Z., and Reed, L.J. (1988) Arch. Biochem. Biophys. 262, 574-584.
14. Chernoff, J., Li, H.-C., Cheng, Y.-S.E., and Chen, L.B. (1983) J. Biol. Chem. 258, 7852-7857.
15. Foulkes, J.G., Erickson, E., and Erickson, R.L. (1983) J. Biol. Chem. 258, 431-438.
16. Sturgill, T.W., Ray, B.L., Erickson, R.E., and Maller, J.L. (1988) Nature 334, 715-718.