Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 263-270

INCREASE OF (Ca^{2+}+Mg^{2+})-ATPase ACTIVITY OF RENAL BASOLATERAL MEMBRANE BY PARATHYROID HORMONE VIA CYCLIC AMP-DEPENDENT MEMBRANE PHOSPHORYLATION

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Received December 1, 1987

SUMMARY. Studies were made on the mechanism of the effect of parathyroid homone (PTH) on the activity of (Ca^{2+}+Mg^{2+})-ATPase, a membrane bound Ca^{2+}-extrusion pump enzyme from the basolateral membranes (BLM) of canine kidney (Km for free Ca^{2+} = 1.3 x 10^{-7} M, Vmax = 200 nmol Pi/mg/min). At 1 x 10^{-7} M free Ca^{2+}, both PTH (10^{-7}-10^{-6} M) and cAMP (10^{-6}-10^{-4} M) stimulated (Ca^{2+}+Mg^{2+})-ATPase activity dose-dependently and their stimulatory effects were inhibited completely by 5 μM H-8, an inhibitor of cAMP-dependent protein kinase. PTH (10^{-7} M) also caused 40 % increase in ^{32}P incorporation into the BLM and 5 μM H-8 inhibited this increase too. PTH (10^{-7} M) was found to stimulate phosphorylation of a protein of Mr 9000 by cAMP dependent protein kinase and 5 μM H-8 was found to block this stimulation also. From these results, it is proposed that PTH stimulates (Ca^{2+}+Mg^{2+})-ATPase activity by enhancing its affinity for free Ca^{2+} via cAMP-dependent phosphorylation of a BLM protein of Mr 9000.

Parathyroid hormone (PTH) is important in regulation of biological processes in target organs. The physiological actions of PTH in renal tubular cells are mediated by activation of adenyl cyclase, the enzyme catalyzing the conversion of adenosine triphosphate (ATP) to adenosine 3',5'-cyclic monophosphate (cyclic AMP, cAMP) (1). Furthermore, PTH is reported (2) to affect the activity of (Ca^{2+}+Mg^{2+})-ATPase, a membrane bound Ca^{2+}-extrusion pump enzyme in the basolateral membranes (BLM) of renal proximal tubular cells, which have receptors for this hormone (3,4). Thus PTH may modulate Ca^{2+}-pump activity by stimulating cAMP-dependent phosphorylation of some membrane protein. In the present report, we found that a renal BLM protein of Mr 9000 was a substrate for cAMP-dependent protein kinase and that PTH stimulated the phosphorylation

0006-291X/88 $1.50

of this protein with simultaneous 1.5-fold increase in the affinity of the Ca^{2+}-pump enzyme for free Ca^{2+} without change in the maximal rate of the enzyme reaction. This mechanism suggests that the action of PTH is in part due to regulation of the intracellular Ca^{2+} concentration.

MATERIALS AND METHODS

Human-PTH(1-84) was obtained from Toyojozo Co., Ltd. (Shizuoka, Japan). cAMP dependent protein kinase inhibitor (H-8) was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). [γ-^{32}P]ATP (2000-3000 Ci/mmol) was from Amersham Co. (Arlington Heights, IL). Trifluoperazine (calmodulin antagonist), cAMP and the cAMP-dependent protein kinase catalytic subunit were purchased from Sigma Chemical Co. (St. Louis, MO). Proximal tubular BLM from canine kidney were prepared by the method of Windus, et al. (5). In this BLM preparation, the plasma membrane markers ouabain sensitive (Na^++K^+)-ATPase and (Ca^{2+}+Mg^{2+})-ATPase (785.7 ± 55.2 and 164.2 ± 12.1 nmol/mg/min, n=4, respectively) were respectively 8.5- and 12.1- fold those in the homogenate, while the endoplasmic reticulum marker rotenone-insensitive NADPH cytochrome c reductase, and the mitochondrial marker cytochrome c oxidase had lower activities (12.2 ± 0.38 and 10.2 ± 0.54 pmol/mg/min) than those in the homogenate (16.6 ± 0.16 and 154 ± 4.3 pmol/mg/min , n=4, respectively). The activities of membrane marker enzymes were assayed by reported methods (6-9). (Ca^{2+}+Mg^{2+})-ATPase activity was measured by a modification of the method of Pershadsingh and McDonald (10). The reaction mixture contained 7.2 µg of BLM protein , 50 mM Tris•HCl (pH 7.2), 20 mM NaN_3 [as mitochondrial ATPase inhibitor], 0.1 mM ouabain [as (Na^++K^+)-ATPase inhibitor], 1 mM ATP and the desired submicromolar free Ca^{2+} concentration, adjusted by addition of 185 µM EGTA. The association constants for Ca^{2+}-EGTA and Ca^{2+}-ATP at pH 7.2 were taken as 6.8×10^7 and 8.5×10^3, respectively (11). The reaction was started by adding ATP and after incubation at 37 °C for 30 min, and it was stopped by adding ice-cold trichloroacetic acid. The mixture was centrifuged and the phosphate (Pi) content released into the supernatant was determined by the method of Youngberg and Youngberg (12). (Ca^{2+}+Mg^{2+})-ATPase activity was calculated by subtracting values obtained with EGTA alone from those with Ca^{2+} and EGTA. The BLM were phosphorylated by incubation at 30 °C in 50 µl of medium containing 50 mM Tris•HCl (pH 7.2), 20 mM NaN_3, 0.1 mM ouabain, 1 mM EGTA and 1 mM [γ-^{32}P]ATP. After the reaction, the extent of phosphorylation of the membranes was determined by a reported method (13). Then samples was submitted to SDS polyacrylamide gel electrophoresis, and radioactive bands were identified. SDS polyacrylamide gel electrophoresis was carried out in the buffer system of Laemmli (14). Protein concentrations were determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Fig. 1A shows the dose-dependence on free Ca^{2+} of the high affinity (Ca^{2+}+Mg^{2+})-ATPase activity of BLM from canine kidney. The

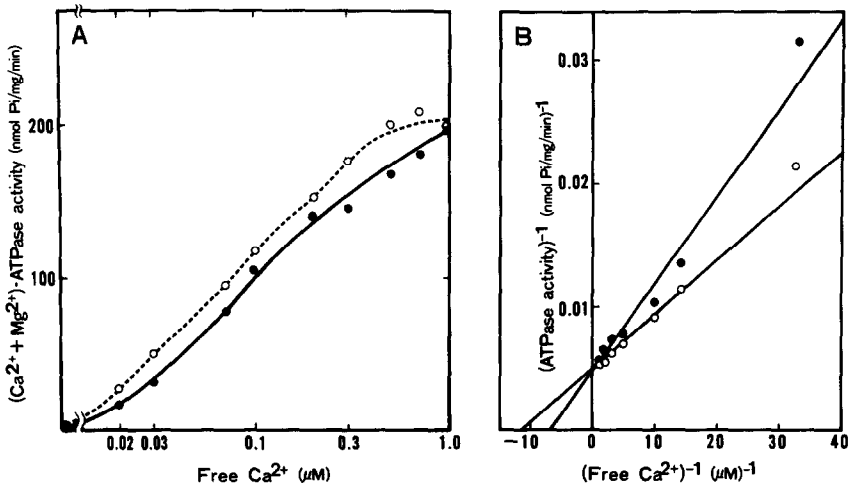

Fig. 1. A. Effect of cAMP on dependence of BLM (Ca^{2+}+Mg^{2+})-ATPase activity on free Ca^{2+}.

Enzyme activity was determined at the indicated free Ca^{2+} concentrations in the absence (●) or presence (○) of 10^{-5} M cAMP.

B. Double reciprocal plot of Fig. 1A.

Km of the enzyme for free Ca^{2+} was 1.3 x 10^{-7} M and the maximum velocity was 200 nmol of Pi/mg/min at 37 °C (Fig. 1B). Trifluoperazine, a calmodulin antagonist, inhibited the enzyme activity in dose-dependently (IC_{50} = 40 μM) (data not shown). These

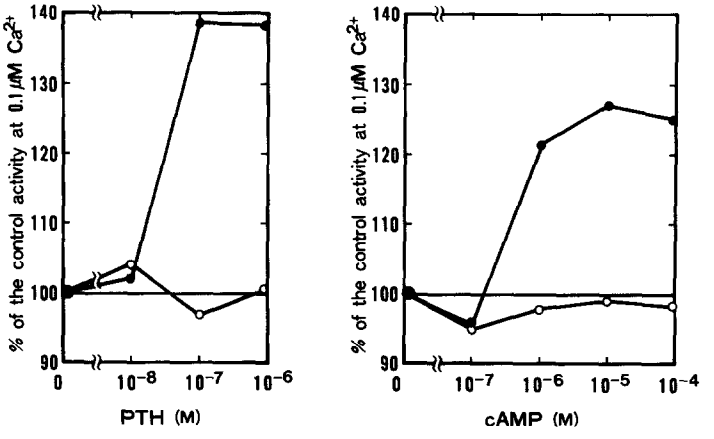

Fig. 2. Effects of human-PTH(1-84) and cAMP on (Ca^{2+}+Mg^{2+})-ATPase activity in canine kidney BLM.

Enzyme activity was estimated at a free Ca^{2+} concentration of 0.1 μM in the absence (●) or presence (○) of 5 μM cAMP-dependent protein kinase inhibitor (H-8). Activities were calculated as percentages of the control activity without additions.

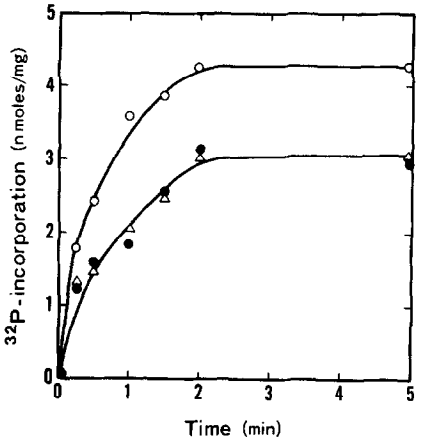

Fig. 3. Time course of phosphorylation of BLM.

BLM (37 µg) were phosphorylated at 30 °C in 50 µl of reaction medium consisting of 50 mM Tris·HCl (pH 7.2), 20 mM NaN_3, 0.1 mM ouabain, 1 mM EGTA and 1 mM [γ-^{32}P]ATP, without (●), or with 10^{-7} M PTH (○), or 10^{-7} M PTH and 5 µM H-8 (cAMP-dependent protein kinase inhibitor) (△).

characteristics were consistent with the reported features of high affinity (Ca^{2+}+Mg^{2+})-ATPase activity (3,4,16).

Next we studied the effect of cAMP on the enzyme activity. On addition of 10^{-5} M cAMP, the Km of the enzyme for Ca^{2+} decreased 1/3 with no change in the maximal velocity (Fig. 1A and 1B). The effects of various concentrations of PTH and cAMP on the (Ca^{2+}+Mg^{2+})-ATPase activity were measured at a Ca^{2+} concentration of 0.1 µM, a concentration in the linear portion of the calcium-dependence curve (Fig. 1A). As shown in Fig. 2, both PTH (10^{-7}-10^{-6} M) and cAMP (10^{-6}-10^{-4} M) stimulated (Ca^{2+}+Mg^{2+})-ATPase activity significantly and dose-dependently. However at 2 µM free Ca^{2+}, which gave the maximal velocity, neither PTH nor cAMP had any effect on the enzyme activity (data not shown).

The stimulatory effects of PTH and cAMP were completely inhibited by addition of 5 µM H-8, a cAMP-dependent protein kinase inhibitor to the reaction mixture. This result suggested that PTH stimulates cAMP-dependent phosphorylation of the BLM. Therefore, we next examined the effect of PTH on phosphorylation of the membranes. Fig. 3 shows the time-course of ^{32}P-incorporation into the BLM. Autophosphorylation of

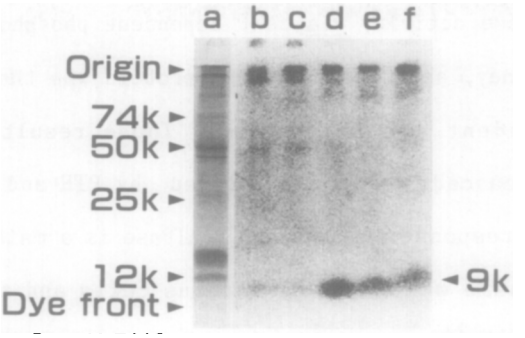

Fig. 4. Autoradiograms of proteins of canine kidney BLM phosphorylated by cAMP-dependent protein kinase

BLM (37 μg) were incubated at 30 °C in the same reaction medium as for Fig. 3 with the following additions; lane (b), none; lane (c), PTH (10^{-7} M) + H-8 (5 μM); lane (d), PTH (10^{-7} M); lane (e), cAMP (10^{-5} M); lane (f), cAMP-dependent protein kinase catalytic subunit (50 μg/ml). After electrophoresis on 15% SDS polyacrylamide gel, gels were stained with Coomassie brilliant blue [lane (a)] or subjected to autoradiography.

BLM reached a plateau in 2 min at 30 °C. 10^{-7} M PTH caused 1.4 fold increase in the phosphorylation of the BLM, and this stimulatory effect was completely inhibited by 5 μM H-8.

Finally for identification of the protein phosphorylated by cAMP-dependent protein kinase, the BLM (37 μg) were incubated with [γ-^{32}P]ATP, and 10^{-7} M PTH, 10^{-5} M cAMP or 50 μg/ml of the cAMP-dependent protein kinase catalytic subunit with or without H-8, and ^{32}P incorporation into proteins was examined by SDS polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 4, a major band of protein of Mr 9000 and some minor bands were autophosphorylated. PTH, cAMP and the cAMP-dependent protein kinase catalytic subunit all stimulated the phosphorylation of this Mr 9000 protein and their stimulatory effects were blocked by H-8. Therefore, PTH stimulated the phosphorylation of the Mr 9000 protein by activating the cAMP-dependent protein kinase present in the BLM preparation.

DISCUSSION

The mechanism by which cells respond to hormones is of great interest. In the present report, we showed that PTH stimulated

$(Ca^{2+}+Mg^{2+})$-ATPase activity *via* cAMP dependent phosphorylation of BLM from canine kidney, and that a Mr 9000 protein was the main substrate of cAMP-dependent protein kinase. These results represent an intracellular cascade of events induced by PTH and resulting in a physiological response. $(Ca^{2+}+Mg^{2+})$-ATPase is a main Ca^{2+}-extrusion pump in the plasma membranes of various cells and tissues (17,18). This enzyme is Ca^{2+}-calmodulin dependent, and Niggli, *et al.* (17) purified it from erythrocytes by calmodulin affinity column chromatography. Furthermore Lamers, *et al.* (6) reported that the $(Ca^{2+}+Mg^{2+})$-ATPase of cardiac sarcolemma was regulated by cAMP through protein kinase as well as by Ca^{2+}-calmodulin. They also found that a cardiac sarcolemma protein of about Mr 9000 is a substrate for both Ca^{2+}-calmodulin dependent kinase and cAMP-dependent protein kinase, and that cAMP dependent phosphorylation caused 1.6-fold increase in the affinity of the Ca^{2+}-pump enzyme for Ca^{2+} without changing its maximal rate. In human blood platelets also, stimulation of Ca^{2+} uptake is accompanied by cAMP-dependent phosphorylation of a membrane protein of about Mr 22000 (19,20). Thus the response to cAMP of stimulation of Ca^{2+}-extrusion pump in renal tubular BLM is like those in cardiac sarcolemma and platelets.

In this work we showed that PTH stimulated cAMP-dependent phosphorylation of the BLM through protein kinase and also activated $(Ca^{2+}+Mg^{2+})$-ATPase activity. This result strongly suggested that PTH regulates Ca^{2+}-pump activity through its receptor. In fact, recently both PTH and cAMP were reported to decrease the intracellular Ca^{2+} concentration in renal proximal tubular cells (21). Therefore, the cellular effect of PTH was partly mediated by change in the intracellular Ca^{2+} concentration.

The most interesting example of a hormone mediated intracellular sequential process is that mediated by phospholamban in the cardiac sarcoplasmic reticulum (SR) reported by Tada, *et al.* (22-24). They

showed that the phosphorylation of a protein of Mr 22000, cardiac SR phospholamban, catalyzed by cAMP-dependent protein kinase, augmented (Ca^{2+}+Mg^{2+})-ATPase activity. The resulting increase in the rate of Ca^{2+} uptake by the SR may account for the increased rate of relaxation of heart muscle in response to epinephrine. Like cardiac muscle, all slow skeletal muscles (25,26), platelets (20), aortic microsomes (27) and Oddi's sphincter (28) also show increased Ca^{2+} uptake in response to epinephrine, and phospholamban-like proteins have been found in these tissues. Recently, Fujii, et al. (29) reported that an intresting feature of phospholamban was the reversible temperature-dependent interconversion of high and low molecular weight forms on SDS polyacrylamide gel electrophoresis. On SDS at 50-70 °C, phospholamban gave three intermediate bands between the protomer band (PN_L) of Mr 6000 and holoprotein band (PN_H) of Mr 26000. The membrane protein of cardiac salcolemma that is a substrate for the cAMP dependent protein kinase also showed temperature-dependent change of mobility on SDS polyacrylamide gel electrophoresis (6). However, the Mr 9000 protein found in the present study, did not show any change in mobility on SDS polyacrylamide gel electrophoresis at temperatures of 4 °C and 100 °C (data not shown). Therefore it is unlikely that this Mr 9000 protein in the BLM is a phospholamban-like protein. Moreover, at present we do not know whether PTH stimulates phosphorylation of BLM in vivo.

ACKNOWLEDGMENTS

This work was supported by grants from "the Research Program on Cell Calcium Signals in the Cardiovascular System" and from the Ministry of Education, Science and Culture of Japan (No. 62570393). We thank Miss Chiaki Nishi for technical assistance in (Ca^{2+}+Mg^{2+})-ATPase assay and Dr. M. Tada, First department of Medicine, Osaka University, for reading this manuscript.

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