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Phosphorylation of the catalytic subunit of Na^+,K^+-ATPase inhibits the activity of the enzyme

(cAMP-dependent protein kinase/protein kinase C/ion pump/sodium pump)

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ABSTRACT **We have examined two distinct protein kinases, cAMP-dependent protein kinase and protein kinase C, for their ability to phosphorylate and regulate the activity of three different types of Na^+,K^+-ATPase preparation. cAMP-dependent protein kinase phosphorylated purified shark rectal gland Na^+,K^+-ATPase to a stoichiometry of approximately 1 mol of phosphate per mol of α subunit. Protein kinase C phosphorylated purified shark rectal gland Na^+,K^+-ATPase to a stoichiometry of approximately 2 mol of phosphate per mol of α subunit. The phosphorylation by each of the kinases was associated with an inhibition of Na^+,K^+-ATPase activity of about 40–50%. These two protein kinases also inhibited the activity of a partially purified preparation of Na^+,K^+-ATPase from rat renal cortex and the activity of Na^+,K^+-ATPase present in preparations of basolateral membrane vesicles from rat renal cortex.**

Na^+,K^+-ATPase, the ion pump responsible for maintaining Na^+ and K^+ gradients in eukaryotic cells, regulates resting membrane potential as well as Na^+-gradient-driven transport of H^+, Ca^{2+}, glucose, and a variety of amino acids and biogenic amines. In this way, it plays a vital role in the functioning of virtually all cells and tissues. Thus, it is understandable that a major portion of the energy expended by most tissues is attributable to the activity of this pump (1). Although there is much evidence indicating that the Na^+,K^+-ATPase is dynamically regulated, it has generally been believed that this regulation is achieved by alterations in the concentration of intracellular Na^+ (2, 3). It was found, using intact cell preparations, that phorbol esters (4), dibutyryl cAMP (5), and phospho-DARPP-32 (the phosphorylated form of the dopamine- and cAMP-regulated phosphoprotein of M_r 32,000), a protein phosphatase inhibitor (6), inhibited Na^+,K^+-ATPase activity, raising the possibility that this ion pump is subject to regulation by phosphorylation/dephosphorylation. However, those studies (4–6) did not address the question of whether such regulation might reflect a direct action on the pump—i.e., whether the pump itself was a substrate for protein kinases and phosphatases. We have now examined two distinct protein kinases, cAMP-dependent protein kinase and protein kinase C, for their ability to phosphorylate and regulate the activity of three different types of Na^+,K^+-ATPase preparation.

MATERIALS AND METHODS

Shark rectal gland Na^+,K^+-ATPase was purified by differential centrifugation and SDS extraction as described (7). The catalytic subunit of cAMP-dependent protein kinase (8) and protein kinase C (9) were purified as described. Phosphorylation assays were carried out at 23°C in a reaction volume of 100 μl containing Hepes (50 mM, pH 7.6), $MgCl_2$ (10 mM), EGTA (1 mM), Na^+,K^+-ATPase (20 μg/ml), [γ-^{32}P]ATP (100 μM, 3000 Ci/mmol; 1 Ci = 37 GBq). Reactions were initiated by the addition of ATP. After 3 min, reactions were terminated by the addition of SDS-stop solution, samples were boiled for 3 min, and proteins were analyzed by one-dimensional SDS/polyacrylamide gel electrophoresis using the method of Laemmli (10). Gels were stained with Coomassie brilliant blue, destained, dried, and autoradiographed. With the autoradiogram as a guide, the ^{32}P-labeled α subunit was excised from the gel and radioactivity was measured by liquid scintillation counting.

For phosphopeptide mapping of Na^+,K^+-ATPase, gel pieces containing ^{32}P-labeled Na^+,K^+-ATPase were cut from the dried gel, washed, and incubated with trypsin. Two-dimensional tryptic phosphopeptide mapping was performed as described (11). Briefly, aliquots of digests were spotted in the middle of the plate, 4 cm from the bottom, and initially separated by horizontal electrophoresis at pH 3.5 in 10% acetic acid/1% pyridine until the dye front migrated 7 cm. Chromatography was performed in the vertical dimension in 1-butanol/acetic acid/water/pyridine (15:3:12:10, vol/vol). Phosphopeptides were visualized by autoradiography. The tryptic digests were hydrolyzed with 6 M HCl and phospho amino acid analysis was performed as described (12).

Na^+,K^+-ATPase activity was measured as the rate of formation of inorganic [^{32}P]phosphate from [γ-^{32}P]ATP, in the absence and presence of ouabain, as described (13).

RESULTS

Incubation of a highly purified preparation of shark rectal gland Na^+,K^+-ATPase with the catalytic subunit of cAMP-dependent protein kinase resulted in the phosphorylation of the α subunit, but not the β subunit, of the enzyme (Fig. 1 *Left*). This phosphorylation of the enzyme was prevented by the presence of PKI-(5–24)-amide (100 μg/ml), a specific peptide inhibitor of cAMP-dependent protein kinase. Protein kinase C also catalyzed the phosphorylation of the enzyme, and this phosphorylation was dependent on the presence of Ca^{2+} in the incubation mixture (Fig. 1 *Right*).

Phosphorylation of the shark rectal gland enzyme by cAMP-dependent protein kinase and by protein kinase C was determined as a function of incubation time. The final stoichiometries were approximately 1.0 and 2.0 mol of phosphate incorporated per mol of α subunit, for cAMP-dependent protein kinase (Fig. 2 *Upper*) and protein kinase C (Fig. 2 *Lower*), respectively. The K_m values for phosphorylation by

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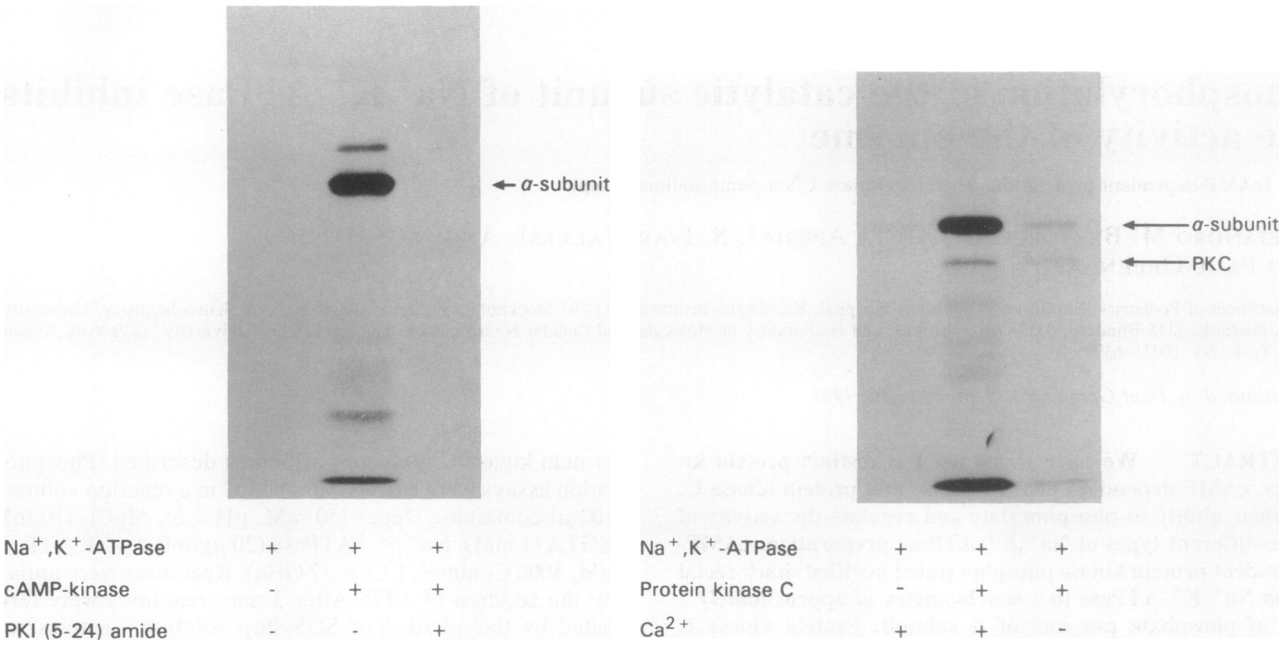

FIG. 1. Phosphorylation of the α subunit of shark rectal gland Na^+,K^+-ATPase by cAMP-dependent protein kinase or protein kinase C. (*Left*) Na^+,K^+-ATPase was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (60 μg/ml) in the absence or presence of the specific peptide inhibitor of cAMP-dependent protein kinase, PKI-(5–24)-amide (100 μg/ml), as indicated; Triton X-100 (0.1%) was present in all assays. (*Right*) Na^+,K^+-ATPase was phosphorylated by protein kinase C (30 μg/ml) in the absence or presence of $CaCl_2$ (1 mM), as indicated. Only the α subunit of the ATPase was phosphorylated. The background labeling is of impurities in the ATPase preparation and not of the β subunit. The position of autophosphorylated protein kinase C is indicated (PKC).

cAMP-dependent protein kinase and protein kinase C were each about 0.1–0.2 μM.

Two-dimensional phosphopeptide maps of the phosphorylated α subunit indicated that a single peptide was phosphorylated by cAMP-dependent protein kinase (Fig. 3*A*), which was distinct from the two peptides that were phosphorylated by protein kinase C (Fig. 3*B*). Phospho amino acid analysis of the holoprotein and the phosphopeptides indicated that cAMP-dependent protein kinase phosphorylated only serine residues, whereas protein kinase C phosphorylated both serine and threonine residues (data not shown).

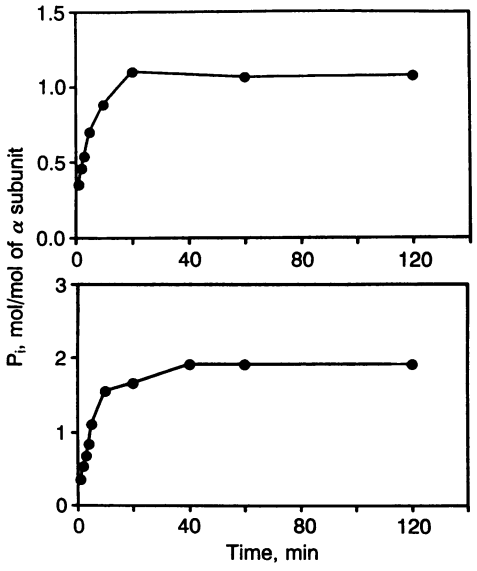

FIG. 2. Phosphorylation of the α subunit of shark rectal gland Na^+,K^+-ATPase by cAMP-dependent protein kinase (*Upper*) or protein kinase C (*Lower*), as a function of incubation time. Phosphorylation assays were carried out as described in *Materials and Methods* except that the incubation time was varied.

Phosphorylation of the Na^+,K^+-ATPase by cAMP-dependent protein kinase, to a stoichiometry of 1 mol of phosphate per mol of α subunit, was associated with 40% inhibition of enzyme activity (Fig. 4 *Left*). Phosphorylation of the enzyme by protein kinase C, to a stoichiometry of 1.8 mol of phosphate per mol of α subunit, was associated with 50% inhibition (Fig. 4 *Right*).

cAMP-dependent protein kinase and protein kinase C also inhibited partially purified Na^+,K^+-ATPase from rat renal cortex. Maximal inhibitions of 58 ± 2% (n = 3) for cAMP-dependent protein kinase and 35 ± 7% (n = 4) for protein kinase C were observed, under the experimental conditions described in *Materials and Methods*. Similar effects of cAMP-dependent protein kinase and of protein kinase C on the activity of Na^+,K^+-ATPase were observed using preparations of basolateral membrane vesicles, prepared as described (14), from rat renal cortex.

DISCUSSION

Previous efforts by a number of research groups to demonstrate effects of phosphorylation on the activity of Na^+,K^+-ATPase were unsuccessful. We have observed apparent substrate inhibition of phosphorylation at concentrations of Na^+,K^+-ATPase as low as 1 μM. We attribute this inhibition of ATPase, at least in part, to traces of SDS, which had been added during the solubilization procedure and remained upon purification of the enzyme. The addition of Triton X-100 reduced the apparent substrate inhibition and accelerated the rate of phosphorylation of the purified enzyme by cAMP-dependent protein kinase. The inability of previous investigators to demonstrate an effect of phosphorylation on the activity of Na^+,K^+-ATPase is most likely attributable to the low maximal stoichiometry of phosphorylation (e.g., 0.125 mol of phosphate per mol of α subunit; ref. 15) achieved previously. The low K_m values for phosphorylation of the pump by cAMP-dependent protein kinase and protein kinase C, the stoichiometric phosphorylation of the pump by the two

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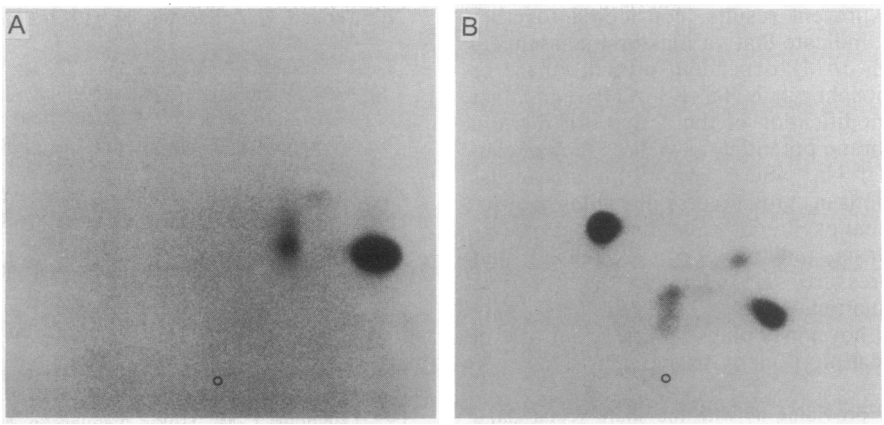

FIG. 3. Two-dimensional phosphopeptide mapping of Na^+,K^+-ATPase phosphorylated by cAMP-dependent protein kinase for 5 min (*A*) or by protein kinase C for 10 min (*B*). Electrophoresis was performed in the horizontal direction (positive electrode, left) and chromatography was performed in the vertical direction.

kinases, and particularly the inhibition of the pump activity by the two kinases all argue strongly that phosphorylation of the pump by the two kinases is of physiological significance and not an *in vitro* artifact. In support of this idea, preliminary evidence indicates that protein phosphatase 1, protein phosphatase 2a, and protein phosphatase 2b dephosphorylated and reactivated Na^+,K^+-ATPase that had been phosphorylated and inhibited by either cAMP-dependent protein kinase or protein kinase C (Y. Yamagata, M. Pessin, A.M.B., A.A., A.C.N., and P.G., unpublished data).

The localization of the amino acid residues of Na^+,K^+-ATPase that are phosphorylated by cAMP-dependent protein kinase and by protein kinase C remains to be determined. The deduced amino acid sequences of several isoforms of the α subunit of Na^+,K^+-ATPase from adult rat tissue have been reported (16, 17). The sequence Lys-Thr-Arg-Arg-Asn-Ser of the α subunit is present in all three isoforms (e.g., at amino acid residues 938–943 of α_1), in a region believed to be located intracellularly. The serine represents a consensus site for cAMP-dependent protein kinase and the threonine represents a consensus site for protein kinase C. Since the consensus sites found in the α_1, α_2, and α_3 isoforms of Na^+,K^+-ATPase are identical, it seems likely that the regulatory phenomena described here apply to all three isoforms.

A number of first messengers, including dopamine (18–20), prostaglandin E_2, endothelin (21), adenosine (22), and insulin and norepinephrine (23), some of which have been associated with alterations in the formation of cAMP and diacylglycerol, have been reported to regulate Na^+,K^+-ATPase activity in intact cells. The results obtained in the present investigation suggest a molecular mechanism for the regulation of Na^+,K^+-ATPase by neurotransmitters and hormones. Thus, neurotransmitters that activate cAMP-dependent protein kinase or protein kinase C would be expected to stimulate the phosphorylation, and inhibit the activity, of Na^+,K^+-ATPase.

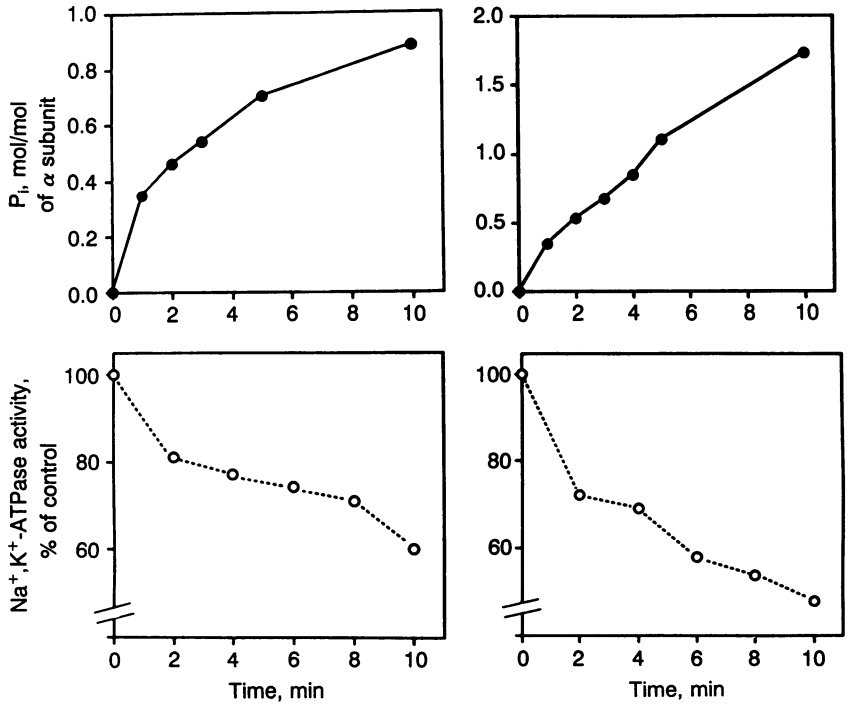

FIG. 4. Phosphorylation of Na^+,K^+-ATPase is associated with inhibition of enzyme activity. A purified preparation of shark rectal gland was phosphorylated in the presence of either cAMP-dependent protein kinase (*Left*) or protein kinase C (*Right*) for the indicated times, and the stoichiometry of phosphorylation (*Upper*) and the Na^+,K^+-ATPase activity (*Lower*) were determined. The effects of cAMP-dependent protein kinase and protein kinase C on Na^+,K^+-ATPase activity were abolished in the presence of PKI-(5–24)-amide and the absence of Ca^{2+}, respectively (*cf.* Fig. 1) (data not shown).

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In conclusion, the present results, considered together with previous studies, indicate that various first messengers can act either through cAMP-dependent protein kinase or protein kinase C to phosphorylate Na^+,K^+-ATPase in intact cells. The resultant modification of the activity of this ion pump alters the membrane potential, as well as the concentrations of intracellular Na^+ and K^+ in all affected cells. Consequently, alterations occur in diverse physiological processes, such as neuronal excitability, uptake of neurotransmitters, Na^+ homeostasis, and control of extracellular fluid volume and blood pressure, depending on the cell type affected. Clearly, regulation of Na^+,K^+-ATPase activity by neurotransmitters and hormones plays an important role in the physiological regulation of many tissues.

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