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Original Article

Phosphorylation of the Na,K-ATPase by Ca,Phospholipid-Dependent and *c*AMP-Dependent Protein Kinases. Mapping of the Region Phosphorylated by Ca,Phospholipid-Dependent Protein Kinase

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Ca,phospholipid-dependent (PKC) and *c*AMP-dependent (PKA) protein kinases phosphorylate the α-subunit of the Na,K-ATPase from duck salt gland with the incorporation of 0.3 and 0.5 mol ^{32}P/mol of α-subunit, respectively. PKA (in contrast to PKC) phosphorylates the α-subunit only in the presence of detergents. Limited tryptic digestion of the Na,K-ATPase phosphorylated by PKC demonstrates that ^{32}P is incorporated into the N-terminal 41-kDa fragment of the α-subunit. Selective chymotrypsin cleavage of phosphorylated enzyme yields a 35-kDa radioactive fragment derived from the central region of the α-subunit molecule. These findings suggest that PKC phosphorylates the α-subunit of the Na,K-ATPase within the region restricted by C_3 and T_1 cleavage sites.

KEY WORDS: Na,K-ATPase; protein kinase C; protein kinase A; protein phosphorylation; proteolysis.

INTRODUCTION

The Na,K-dependent ATPase is an integral membrane protein that couples ATP hydrolysis to the transport of Na^+ and K^+ across the plasma membrane. It was shown that the transport activity of the Na,K-ATPase *in vivo* is regulated by phorbol esters and *c*AMP (Vasilets *et al.*, 1990, 1991; Vasilets and Schwarz, 1992) which are well-known activators of protein kinase C (PKC) and protein kinase A (PKA), respectively. It was also demonstrated that the α-subunit of the Na,K-ATPase is phosphorylated *in vivo* in Friend erythroleukemia cells by endogenous *c*AMP-independent protein kinase (Yeh *et al.*, 1983; Ling and Cantley, 1984) and *in vitro* by purified PKC and PKA (Mardh, 1988; Lowdens *et al.*, 1990; Chibalin *et al.*, 1991; Bertorello *et al.*, 1991). However, many important questions concerning the process of phosphorylation by these two protein kinases were not addressed in these papers. For example, though phosphorylation of serine and threonine residues has been reported for duck salt gland and shark rectal gland preparations (Lowdens *et al.*, 1990; Bertorello *et al.*, 1991), the localization of these phosphorylation sites within the α-subunit is still unknown.

In this work we have characterized and compared the phosphorylation of Na,K-ATPase from duck salt gland by PKA and PKC under different conditions. Using the method of selective proteolytic digestion, we have also tried to identify the region of the α-subunit phosphorylated by PKC.

METHODS

The Na,K-ATPase, of specific activity 1600–2000 μmol P_i/mg protein/h was prepared from duck salt gland as described previously (Boldyrev *et al.*, 1981). It is a membrane-bound complex containing 90–95% of proteins corresponding to the α- and β-subunits of the enzyme. PKC was purified from rat

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brain (Vorotnikov *et al.*, 1988). PKA from bovine heart and *c*AMP were from Sigma, and trypsin and α-chymotrypsin were from Serva. [γ-^{32}P]ATP was obtained from Amersham. Ingredients for polyacrylamide gel electrophoresis were from Bio-Rad and molecular weight calibration protein markers (LMW-Kit) were purchased from Pharmacia. Phosphorylation of 5 μg of Na,K-ATPase preparation was performed at 30°C in 40 μl of buffer I (25 mM Tris-phosphate, pH 7.3, and 250 μM ouabain) or buffer II (30 mM Tris-chloride, pH 7.3, 120 mM NaCl, and 10 mM KCl) for 30–60 min. The incubation medium also contained 50 μM [γ^{32}-P]ATP (500–100 cpm/pmol), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.17 mM EDTA, and other ingredients which are indicated in the figure legends. After preincubation of Na,K-ATPase in buffer I or II for 20 min, the phosphorylation was started by the addition of labeled ATP. Reaction was stopped by Laemmli sample buffer. Samples were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gel (Laemmli, 1970). Gels were stained with Coomassie blue R-250 and phosphoproteins were visualized by autoradiography. The stoichiometry of phosphorylation of the α-subunit by protein kinases was estimated according to Lowndes *et al.* (1990). Incorporation of ^{32}P in the fraction of operating enzyme molecules was calculated assuming the specific activity of the Na,K-ATPase preparation given above. Protein content in the stained peptide bands was measured as described by Walton *et al.* (1987).

Thermoinactivation of the Na,K-ATPase was performed for 10 min at 56°C. Solubilization of the enzyme with Triton X-100 or other detergents was done at room temperature for 20 min. Proteolysis of the purified Na,K-ATPase before or after phosphorylation by PKC was performed with trypsin or chymotrypsin (Jorgensen and Farley, 1988). Protein concentration was measured by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Phosphorylation of the Na,K-ATPase from duck salt gland was studied under the following conditions: (1) native enzyme in the presence of Na^+, K^+, ATP, and Mg^{2+} (buffer II); (2) enzyme–ouabain complex (buffer I); (3) native enzyme or enzyme–ouabain complex solubilized by 0.2% Triton X-100; (4) thermoinactivated Na,K-ATPase.

Phosphorylation of the Na,K-ATPase by PKC

As is shown in Fig. 1, PKC phosphorylates a protein with relative molecular mass identical to that for the α-subunit of the Na,K-ATPase (about 100 kDa). Radioactivity was not found in the region of the gel where the β-subunit of Na,K-ATPase was stained (a protein with a relative molecular mass of about 55 kDa). Phosphorylation of the α-subunit is facilitated by Ca^{2+} ions (compare lanes 2 and 3 in Fig. 1A). The addition of phospholipids (PL) does not increase the incorporation of ^{32}P in the α-subunit (lanes 3 and 4), presumably due to the presence of endogenous phospholipids in the preparation of Na,K-ATPase. The α-subunit of native enzyme in the presence of Na^+, K^+, and Mg^{2+} is not as effectively phosphorylated as the enzyme–ouabain complex (Fig. 1, lanes 3). This may result from partial hydrolysis of the labeled ATP by the operating enzyme. Calculation of the amount of the labelled ATP that can be consumed by the operating enzyme (5 μg of preparation with a specific activity of 2000 μmol P_i/mg protein for 60 min of incubation) gives a value of about 20% of [γ^{32}-P]ATP. Since the increase in phosphorylation signal in the presence of ouabain can exceed 20%, there might be at least two reasons for the increase of the ^{32}P labelling. In addition to reduced ATP consumption during ATPase activity, ouabain fixes the enzyme to the E_2 conformation, which could be favorably phosphorylated by the protein kinases. Another possible explanation is that the binding of ouabain to the Na,K-ATPase changes the conformation of the α-subunit, which in turn makes the phosphorylation site more accessible for PKC.

The highest level of phosphorylation of the α-subunit by PKC was observed after thermoinactivation of Na,K-ATPase (Fig. 1A, lane 6). This suggests that phosphorylation sites are more accessible for PKC when the α-subunit is subjected to thermoinduced conformational changes.

Phosphorylation of the Na,K-ATPase by PKA

PKA also phosphorylates the α-subunit of the Na,K-ATPase. However, the phosphorylation takes place only after solubilization of Na,K-ATPase with 0.2% Triton X-100 (Fig. 2A, lanes 3 and 4 and Fig. 2B, lanes 1 and 2) or with other detergents like octylglucoside, CHAPS, and NP-40 (data not shown). In corporation of ^{32}P in the solubilized α-subunit was not detected when PKA or *c*AMP were omitted (compare Fig. 2A, lanes 2, 4, 5 and Fig. 2B, lanes 1 and 3).

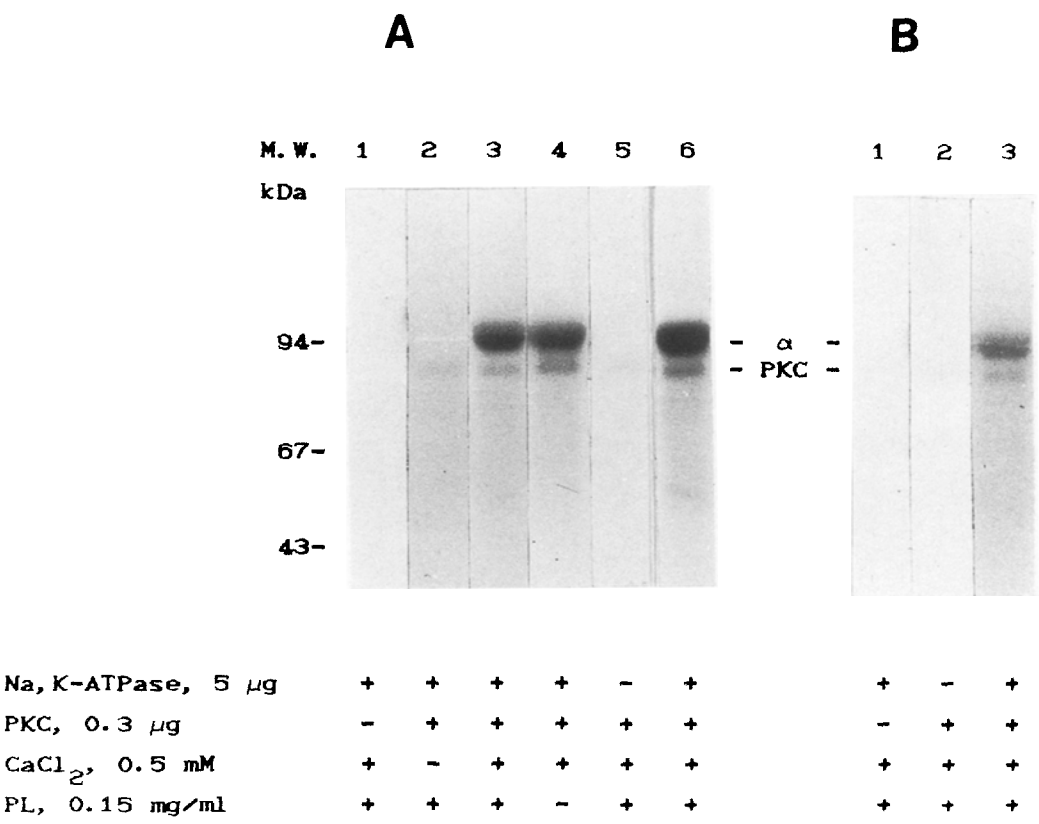

Fig. 1. Phosphorylation of the Na,K-ATPase from duck salt gland by PKC. Native (lanes 1–5) or thermoinactivated (lane 6) Na,K-ATPase was phosphorylated as described in Methods in the presence (A) and in the absence (B) of ouabain with (+) or without (−) ingredients indicated below the figure. ^{32}P-Containing proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to the Laemmli method and then visualized by autoradiography. The α-phosphorylated form of the α-subunit and the PKC-autophosphorylated form of PKC are indicated.

Again, higher ^{32}P-labeling was obtained when the enzyme was treated by ouabain before incubation with PKA (Fig. 2A, lane 4 and Fig. 2B, lane 2). In contrast to the effect of PKC, phosphorylation by PKA was reduced after thermoinactivation of the Na,K-ATPase (Fig. 2, lanes 4 and 6). The fact that phosphorylation by PKA takes place only in the presence of small amounts of Triton or another detergent raises the question of the physiological relevance of phosphorylation. To answer this question, experiments with phosphorylation of Na,K-ATPase *in vivo* are necessary.

Quantitative Estimation of ^{32}P-Incorporation into the α-Subunit

Phosphorylation of the α-subunit by both protein kinases was studied under optimal conditions: in the presence of ouabain (buffer 1) and with the addition of Triton X-100 (when phosphorylation by PKA was investigated). Figure 3 shows the time course of ^{32}P-incorporation into the α-subunit. The maximal values of ^{32}P-incorporation were reached in 60–80 min after start of the reaction and consisted of 0.3 and 0.5 mol/mol of α-subunit for PKC and PKA, respectively. A small gradual decrease of ^{32}P-incorporation observed in more than 1 h after start of the reaction may be due to the effect of endogenous phosphoprotein phosphatases that can account for the inability to achieve equimolar incorporation of ^{32}P into the α-subunit. Another reason is that Na,K-ATPase might be purified from the tissue already being partially phosphorylated.

Identification of the Phosphorylation Region of the α-Subunit

To identify the region of the α-subunit phosphorylated by PKC, the Na,K-ATPase was subjected to controlled cleavage with trypsin or α-chymotrypsin according to Jorgensen and Farley (1988). It was shown that digestion of pig kidney enzyme in the

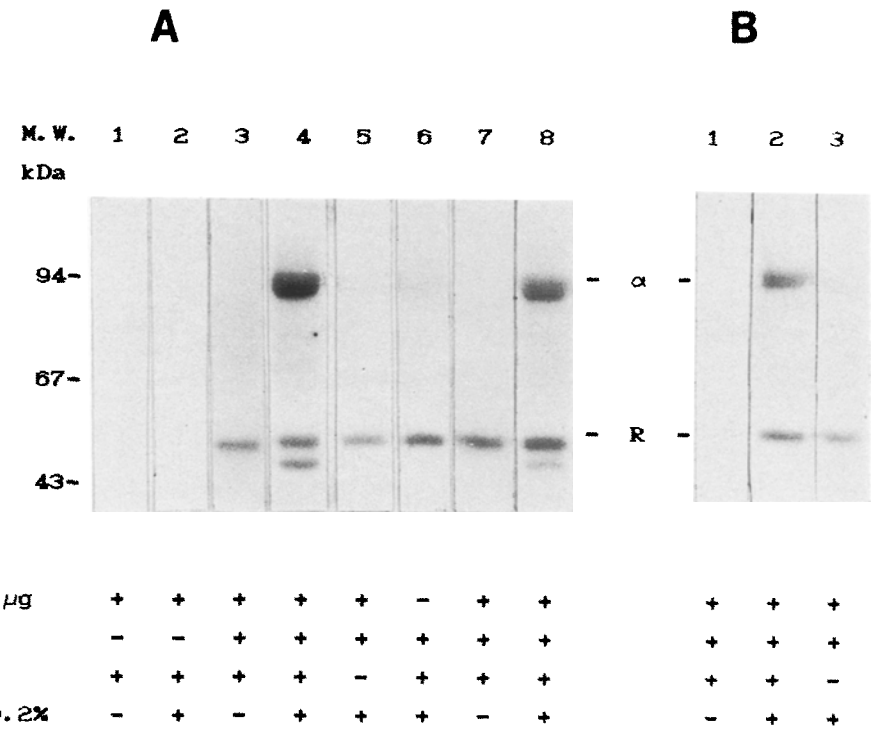

Fig. 2. Phosphorylation of the Na,K-ATPase from duck salt gland by PKA. Native (lanes 1–6) and thermoinactivated (7–8) Na,K-ATPase was phosphorylated in the presence (A) and in the absence (B) of ouabain with (+) or without (−) the ingredients indicated below the figure and then analyzed as described in the legend to Fig. 1.

presence of ouabain by chymotrypsin splits off the 77-kDa fragment (C_3 cleavage site) from the C-terminus of the α-subunit. (Jorgensen and Farley, 1988). Then chymotrypsin cuts the 77-kDa polypeptide into the 35-kDa fragment and the C-terminal 40-kDa fragment. Digestion of the α-subunit in the E_2conformation with trypsin yields the N-terminal polypeptide with molecular mass 41–45-kDa and the C-terminal 58-kDa fragment (T_1 cleavage site).

In our experiments with nonphosphorylated duck salt gland Na,K-ATPase the proteolytic pattern obtained after trypsin and chymotrypsin treatment was similar to those described for pig kidney enzyme by Jorgensen and Farley (1988). The same pattern was obtained for Na,K-ATPase phosphorylated by PKC. Digestion of the phosphorylated enzyme in the E_2-conformation in the presence of ouabain with trypsin produces fragments of molecular mass 41 and 58-kDa (Fig. 4A, lane 2). Chymotrypsin splits off the 77-kDa fragment from the α-subunit, which further degrades to the 32–35-kDa and the 40-kDa fragments (Fig. 4A, lanes 2).

Nearly all ^{32}P incorporated into the α-subunit after incubation with PKC was found in the tryptic fragment with molecular mass of 41 kDa or in the chymotryptic 32–35 fragment (Fig. 4, lanes 4). Superposition of the data of these two experiments restricts the phosphorylation region to a polypeptide with molecular mass of approximately 19 kDa located between the C_3 and T_1 cleavage points. In accordance with the proteolytic cleavage map identified for pig kidney enzyme with known amino acid sequence (Jorgensen and Collins, 1986), this polypeptide is placed between Leu 266 and Arg 438. Asp 369, which is phosphorylated during the pump cycle, is also located in this region. The amino acid sequence for the duck salt gland α-subunit is still unknown. Because the regions centered around Leu 266 and Arg 438 are highly conservative, we suggest that the segment phosphorylated by PKC in the enzyme from duck salt gland is restricted by the same amino acids.

The proteolytic fragments of the Na,K-ATPase solubilized by Triton X-100 were not identical to those described by Jorgensen. Therefore we cannot use this approach to identify the region of the α-subunit that is phosphorylated by PKA.

Our data demonstrate that the α-subunit of the Na,K-ATPase from duck salt gland is a target protein

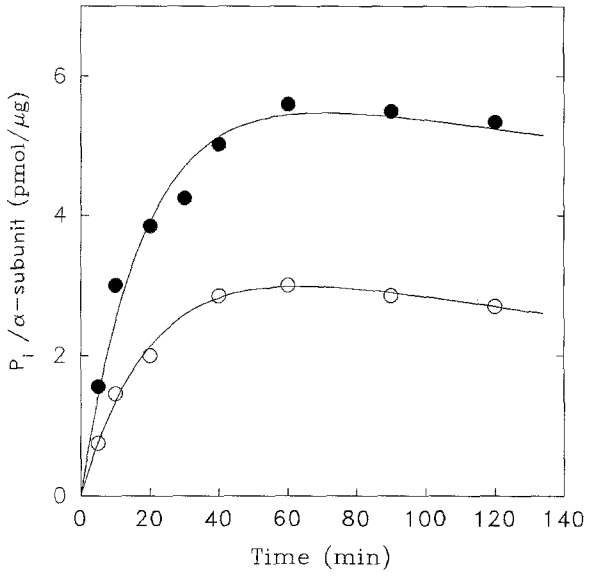

Fig. 3. Time course of ^{32}P-incorporation into the α-subunit of Na,K-ATPase by PKC (○) and by PKA (●). Phosphorylation of the enzyme by PKC was performed for different time intervals in buffer I as described in Methods. The incubation medium also contained the ingredients indicated below lane 4 in Fig. 1A. Phosphorylation by PKA was done under the same conditions but in the presence of the ingredients indicated below lane 4 in Fig. 2A. ^{32}P-Containing proteins were analyzed as described in the legend to Fig. 1. Bands corresponding to the α-subunit were cut from the gel, and associated radioactivity was estimated by scintillation counting.

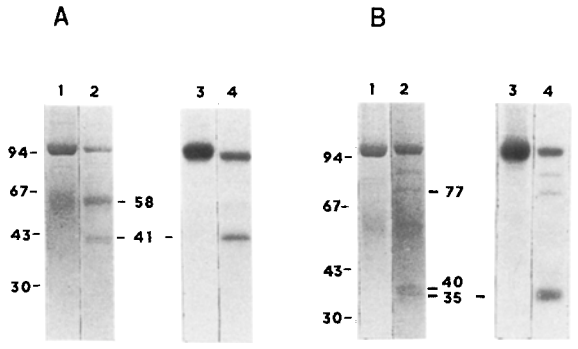

Fig. 4. Proteolysis of the Na,K-ATPase phosphorylated by PKC with trypsin (A) and α-chymotrypsin (B). Coomassie-stained gels (lanes 1 and 2) and autoradiograms of ^{32}P-containing proteins (lanes 3 and 4) after SDS-polyacrylamide gel electrophoresis of phosphorylated Na,K-ATPase before (lanes 1 and 3) and after (lanes 2 and 4) proteolytic digestion. 5 μg of Na,K-ATPase preparation was phosphorylated in buffer I in the presence of 200 μM of ouabain, 0.3 μg PKC, and 0.5 mM $CaCl_2$. PKC was preincubated in the same medium containing "cold" ATP to "chase" the incorporation of ^{32}P in PKC due to autophosphorylation. Phosphorylation was stopped by addition of "cold" ATP in buffer 1 to a final concentration of 1 mM. Then KCl (100 mM), trypsin (A), or chymotrypsin (B) were added with a protein/protease ratio of 50/1 or 6/1, respectively. Proteolysis was performed for 20 min at 37°C. For other details, see Methods.

for either PKC or PKA. Differences in the phosphorylation of thermoinactivated Na,K-ATPase as well as the fact that PKA opposite to PKC phosphorylates the α-subunit only in the presence of detergents suggest that the phosphorylation sites for PKA and PKC are not identical. The region phosphorylated by PKC is restricted by the C_3 and T_1 cleavage sites and is predominantly cytoplasmic. According to the data of Jorgensen (1991) this region of the α-subunit is involved in the E_1–E_2 transition. In light of these observations, the modulation of extracellular K-binding and pump-generated current by activation of PKC and PKA (Vasilets *et al.*, 1990, 1991; Vasilets and Schwarz, 1992) may be attributed to the post-translational modification of functionally important domains within the α-subunit of Na,K-ATPase via their direct phosphorylation.

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