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Phosphorylation of the α-Subunit of Na,K-ATPase from Duck Salt Glands by cAMP-Dependent Protein Kinase Inhibits the Enzyme Activity

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Abstract—Although it was shown earlier that phosphorylation of Na,K-ATPase by cAMP-dependent protein kinase (PKA) occurs in intact cells, the purified enzyme *in vitro* is phosphorylated by PKA only after treatment by detergent. This is accompanied by an unfortunate side effect of the detergent that results in complete loss of Na,K-ATPase activity. To reveal the effect of Na,K-ATPase phosphorylation by PKA on the enzyme activity *in vitro*, the effects of different detergents and ligands on the stoichiometry of the phosphorylation and activity of Na,K-ATPase from duck salt glands (α1β1-isoenzyme) were comparatively studied. Chaps was shown to cause the least inhibition of the enzyme. In the presence of 0.4% Chaps at 1 : 10 protein/detergent ratio in medium containing 100 mM KCl and 0.3 mM ATP, PKA phosphorylates serine residue(s) of the Na,K-ATPase with stoichiometry 0.6 mol P_i/mol of α-subunit. Phosphorylation of Na,K-ATPase by PKA in the presence of the detergent inhibits the Na,K-ATPase. A correlation was found between the inclusion of P_i into the α-subunit and the loss of activity of the Na,K-ATPase.

Key words: Na,K-ATPase, cAMP-dependent protein kinase, phosphorylation, phosphoserine, detergents

Na,K-ATPase (Na-pump) transports Na^+ and K^+ ions across the plasma membrane against the electrochemical gradient using the energy of hydrolysis of ATP. The enzyme is found in various animal cells. It provides for the maintenance of low intracellular Na^+ concentration and high intracellular K^+ concentration. Na,K-ATPase consists of two different polypeptide chains: the catalytic or α-subunit ($M_r \approx 100$ kD) and the β-subunit, which is a glycoprotein (M_r of protein part ≈ 35 kD). Four isoforms of the α-subunit (α1, α2, α3, and α4) and three isoforms of the β-subunit (β1, β2, and β3) of Na,K-ATPase have been identified. All are products of different genes. They are characterized by different distribution in various tissues and organs, and they can be assembled into αβ-dimer in different combinations creating isoenzymes with different properties [1]. The homology of amino acid sequences of α-subunits of different isoforms is quite high: the identity is about 85%. The homology in amino acid sequences of β-subunits is significantly lower, around 40%. The carbohydrate moiety of the β-subunit with molecular mass between 15 and 25 kD has different composition depending on the isoform and tissue type (see review [2]).

Na,K-ATPase activity in various tissues is changes in response to hormones and mediators affecting the intracellular level of cAMP [3-5]. It has been shown that Na,K-ATPase from various tissues including mammalian [6-8] and amphibia kidney [9], shark rectal glands [8, 10], and duck salt glands [11] is phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA). A target site for the phosphorylation is a unique serine residue corresponding to Ser-943 of the Na,K-ATPase α-subunit from rat and *Bufo marinus* kidney [7, 12]. This serine residue is phosphorylated by PKA both *in vitro* [7] and in intact cells [12, 13]. However, in purified preparations of Na,K-ATPase as well as in microsomal fractions enriched in this enzyme, the corresponding serine residue is phosphorylated by PKA only in the presence of a detergent [9-12] or after reconstitution of detergent-solubilized Na,K-ATPase into phospholipid vesicles [8].

A key concern in all studies of reversible phosphorylation control is how phosphorylation affects the enzyme activity. In the case of Na,K-ATPase phosphorylation by

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PKA this problem is not completely solved. First, experiments in which the phosphorylation of Na,K-ATPase by PKA were studied were performed in the presence of Triton X-100, which quickly inactivated the enzyme [9, 10]. Thus, it was difficult to reveal the effect of the phosphorylation on the Na,K-ATPase activity in these experiments. However, Bertorello et al. reported about inhibition of Na,K-ATPase from shark rectal glands after its phosphorylation by PKA in the presence of Triton X-100 without detailed description of the experiment [10]. Later, Cornelius and Logvinenko, using Na,K-ATPase from two different tissues reconstituted into phospholipid vesicles, showed that phosphorylation of Na,K-ATPase from rectal glands by PKA enhanced its transport activity, but phosphorylation of Na,K-ATPase from pig kidney had no effect on this parameter [8]. Because Na,K-ATPase from pig kidney contains the α1-isoform, and Na,K-ATPase from rectal glands contains the α3-isoform, the authors suggested that PKA-mediated phosphorylation affects the activity of various isoforms differently.

The expression of a mutant α1-subunit of Na,K-ATPase in which Ser-943 was substituted by alanine in monkey kidney cells did not reveal the decrease of enzyme activity in response to the addition of pharmacological agents increasing intracellular cAMP level. However, a decrease of Na,K-ATPase activity in response to these agents was observed under these conditions in cells where wild-type α1-subunit was expressed [13]. Finally, the measurement of the activity of Na,K-ATPase from rat [7] and pig [8] kidney phosphorylated by PKA *in vitro* in the presence of Chaps, which did not completely suppress the activity of Na,K-ATPase, has shown that phosphorylation does not influence the enzyme activity. However, in these studies, either the phosphorylation stoichiometry was quite low [7], or the authors did not check this parameter in the experiments where the activity was measured [8]. Therefore, the question whether phosphorylation of Na,K-ATPase by PKA changes the enzyme activity is still open. In the present work, we have found conditions that provide relatively high phosphorylation stoichiometry of purified Na,K-ATPase from duck salt glands by PKA in the presence of the detergent. Under these conditions, the rate of ATP hydrolysis by Na,K-ATPase decreased with increasing the stoichiometry of phosphorylation. We observed a correlation between the loss of Na,K-ATPase activity and the incorporation of $^{32}P_i$ into the α-subunit of the enzyme.

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: EDTA, Tris, ATP disodium salt, ouabain, imidazole, sucrose, NADH, Chaps, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, and histone H1 from Sigma (USA); sodium dodecyl sulfate (SDS) from Serva (Germany); dithiothreitol from Boehringer Mannheim (Germany). The triethylammonium salt of [γ-^{32}P]ATP was purchased from Izotop (Obninsk, Russia). The sodium salt of ATP was converted into the Tris salt by ion-exchange chromatography. The catalytic subunit of PKA (type II) from bovine brain (specific activity about 0.5 μmol/mg protein per min) was kindly provided by Professor E. S. Severin (Laboratory of Enzyme Chemistry, School of Biology, Lomonosov Moscow State University). Some experiments were repeated using the catalytic subunit of PKA from porcine heart purchased from Sigma. All other chemicals were the best grade available from local commercial sources.

Purification of Na,K-ATPase. Na,K-ATPase was purified from the supraorbital salt glands of domestic ducks that were kept for 10 days on a diet with a high NaCl content as described previously [14]. The Na,K-ATPase purification procedure includes extraction of contaminating proteins of the microsomal fraction by treatment with a low concentration of SDS followed by centrifugation on a discontinuous sucrose density gradient. The final fraction (pellet) contains Na,K-ATPase and endogenous lipids bound to the enzyme. Enzyme preparations were stored at −20°C and used within 2-30 days. Final preparations had activities of 15-25 μmol P_i/mg protein per min.

Phosphorylation of purified Na,K-ATPase by PKA. Duck salt gland Na,K-ATPase (10-40 pmol) was preincubated for 10 min at 30°C in 45 μl of solution containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl or KCl, and detergents at concentrations indicated in the figure legends. After preincubation, 10-48 pmol of PKA was added and reaction was started by the addition of 5 μl of [γ-^{32}P]ATP to final concentration 20-300 μM (1,000-2,000 cpm/pmol). Phosphorylation was allowed to proceed for the indicated time and was stopped by the addition of SDS-containing electrophoresis sample buffer (0.5% SDS, 0.2% β-mercaptoethanol, 40 mM Tris-HCl, pH 6.8, 8% sucrose). SDS-PAGE was performed according to Laemmli [15] using 4% stacking and 8.5% separating polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250 and dried. Phosphoproteins were visualized by autoradiography on Hyperfilm™ MP (Amersham).

Protein assay. Protein was assayed by the method of Lowry et al. [16].

Determination of stoichiometry of phosphorylation of Na,K-ATPase α-subunit. The stoichiometry of phosphorylation was estimated by the following method. The gels stained with Coomassie were scanned using an UltroScan XL enhanced laser densitometer (LKB, Sweden). The amount of α-subunit in the band was calculated using a calibration curve (the dependence of the area of the α-subunit band on the gel after staining upon the amount of α-subunit loaded on the lane). The calculations were done using the GelScan XL computer program (LKB).

The content of α-subunit in pmol was calculated taking the value of its molecular mass as 112 kD. The

bands corresponding to phosphorylated α-subunit of Na,K-ATPase were cut out, destained in 40% isopropanol at 56°C, dissolved in 2 ml of 30% H_2O_2 (60°C overnight), and then the amount of incorporated ^{32}P was counted using a Rackbeta-1214 counter (LKB).

Na,K-ATPase activity measurement. Na,K-ATPase activity was measured using the coupled enzyme system previously described [17] at 37°C. Na,K-ATPase (15-40 pmol) was added to 1 ml of incubation medium containing 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 30 mM imidazole, pH 7.5, and 15 and 10 units of lactate dehydrogenase and pyruvate kinase, respectively. The decrease of NADH concentration was monitored by the decrease in absorbance at 340 nm using a Hitachi-200-20 spectrophotometer.

PKA activity determination. PKA activity was measured at 30°C in 50 μl of incubation mixture containing 25 mM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mg/ml histone H1, 48 pmol PKA (catalytic subunit), and 20-300 μM [γ-^{32}P]ATP (1,000-2,000 cpm/pmol). After 5-10 min of incubation, the reaction was stopped by addition of 20 μl of solution containing 0.25 mM EDTA and 50 mM sodium pyrophosphate, and aliquots of the samples (20-25 μl) were spotted on pieces (18 × 18 mm) of Whatman 3MM paper. The paper pieces were washed 3-5 times for 15 min in 12% trichloroacetic acid, 1 time for 5 min in acetone, and dried in air. Radioactivity of the paper pieces was then quantified in 5 ml of toluene fluid by liquid scintillation counter.

Determination of ATP hydrolysis. To determine whether ATP hydrolysis took place under different reaction conditions, the content of $^{32}P_i$ in the incubation medium at the end of the phosphorylation reaction was measured as described previously [18]. The reaction was stopped by the addition of 0.5 ml of stop-solution (10% suspension of activated charcoal in buffer containing 40 mM EDTA, 50 mM KH_2PO_4, pH 7.2) and was shaken for 10 min. Then the samples were centrifuged for 10 min at 5000 rpm, 250-μl aliquots were removed, and the content of $^{32}P_i$ was quantified using liquid scintillation counter.

Phosphoamino acid analysis. The phosphorylated preparations of Na,K-ATPase were separated by SDS-PAGE and then transferred from the gel to an Immobilon-P membrane (Millipore, USA) using an electroblotting procedure. The band corresponding to phosphorylated α-subunit was visualized by autoradiography, cut out, and hydrolyzed in 6 M HCl at 110°C for 4 h. Phosphoamino acid analysis was performed according to the method Boyle et al. [19] using one-dimensional high voltage electrophoresis in the mixture formic acid–acetic acid–water (52 : 29 : 919) at pH 1.9 on thin-layer cellulose plates (Serva).

Each experimental point on the figures is the result of three independent measurements.

RESULTS

Phosphorylation of duck salt gland Na,K-ATPase by PKA. A typical result of SDS-PAGE analysis of duck salt gland Na,K-ATPase phosphorylated by PKA from bovine brain is presented in Fig. 1a. The gel stained with Coomassie R-250 is shown on the left part of this figure. It can be seen that preparations of purified Na,K-ATPase contained mainly two proteins (Fig. 1a, lane *1*): one with molecular mass of about 100 kD is well stained by Coomassie (the α-subunit), and the second one with molecular mass of about 55 kD is seen as a diffuse band which is poorly stained by Coomassie (the β-subunit). The PKA preparation (Fig. 1a, lane *3*) contained a main 42-kD protein component (the catalytic subunit). As can be seen from autoradiography of this gel (Fig. 1b, lane *2*), incubation of Na,K-ATPase with the PKA and [γ-^{32}P]ATP in the presence of 0.2% Triton X-100 resulted in incorporation of ^{32}P into the α-subunit but not into the β-subunit of the enzyme. The catalytic subunit of PKA was phosphorylated both in the presence and absence of Na,K-ATPase (Fig. 1b, lanes *2* and *3*, respectively). No phosphorylation of Na,K-ATPase α-subunit was observed when the PKA (Fig. 1b, lane *1*) or Triton X-100 (data not shown) were not added to the incubation medium. These data are in good agreement with results obtained earlier [7, 9-11]. It should be noted that similar

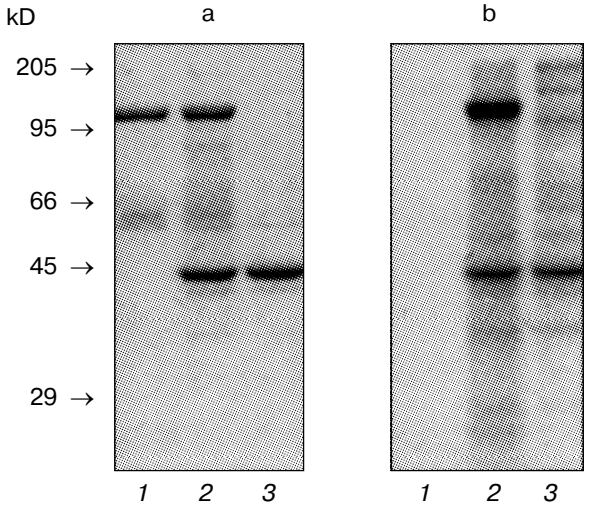

Fig. 1. Phosphorylation of duck salt gland Na,K-ATPse by PKA. a) Gel stained with Coomassie R-250 after separation of phosphorylated proteins of Na,K-ATPase preparations using SDS-PAGE; b) autoradiography of the same gel. Na,K-ATPase (24 pmol), PKA (48 pmol), or both enzymes were incubated for 30 min in the presence of 0.2% Triton X-100 and 100 μM [γ-^{32}P]ATP under conditions described in "Materials and Methods". After termination of the reaction by SDS-containing buffer, the proteins were loaded on gel lanes: *1*) Na,K-ATPase; *2*) Na,K-ATPase + PKA; *3*) PKA.

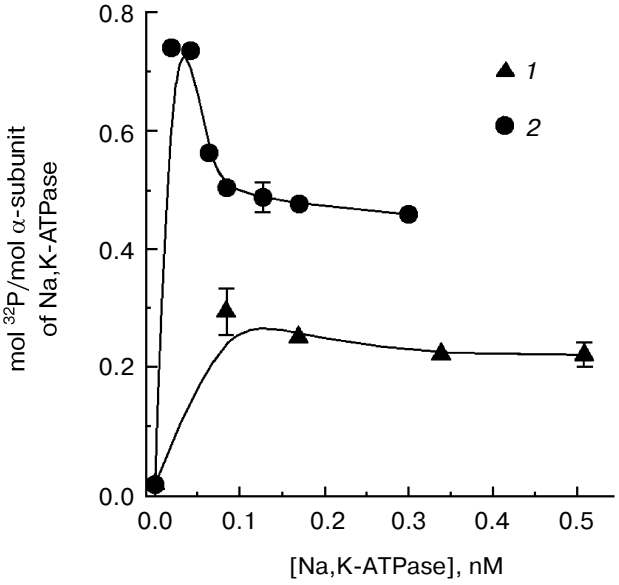

Fig. 2. Dependence of phosphorylation stoichiometry on the concentration of Na,K-ATPase α-subunit in the incubation medium. Phosphorylation was performed in the presence of 0.2% Triton X-100 and 48 pmol of PKA with 20 (*1*) or 100 μM (*2*) [γ-^{32}P]ATP as described in "Materials and Methods".

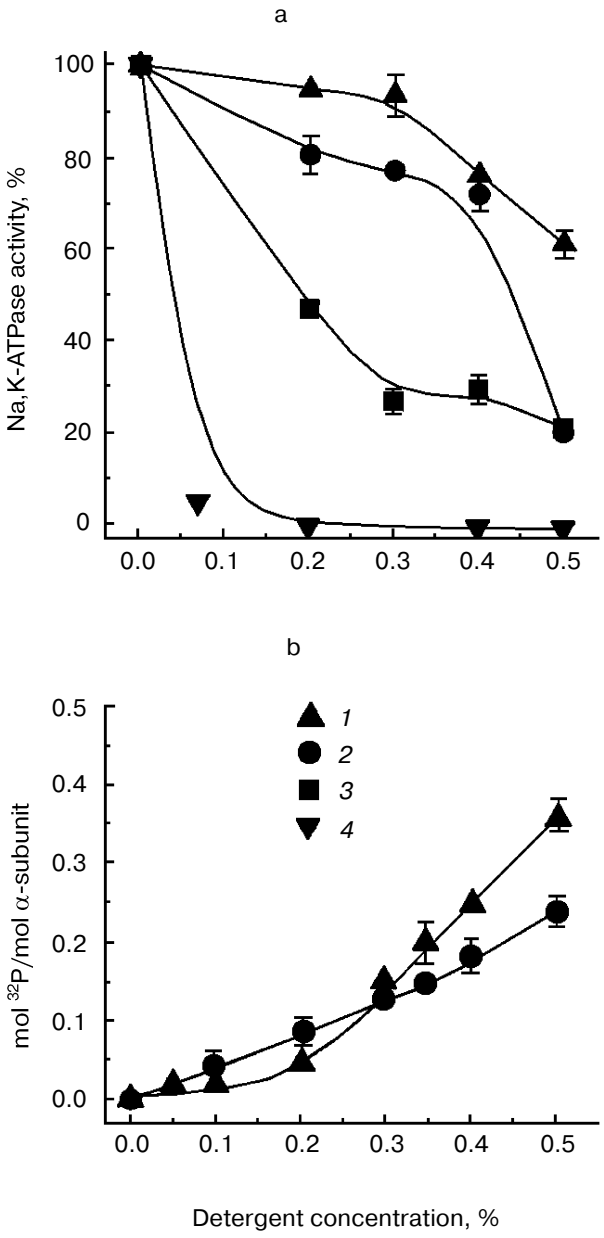

Fig. 3. Effect of different concentrations of Chaps (*1*), *n*-octyl-β-glucopyranoside (*2*), Brij 35 (*3*), and Triton X-100 (*4*) on the activity of duck salt gland Na,K-ATPase (a) and on the stoichiometry of Na,K-ATPase phosphorylation by PKA (b). To measure the activity, 200 pmol of Na,K-ATPase were preincubated for 10 min in the presence of 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl, 25 mM Tris-HCl, pH 7.4, at the indicated concentrations of one of the detergents in a volume of 50 μl. Then 5 μl of mixture was transferred into the incubation medium for the measurement of Na,K-ATPase activity. For the phosphorylation, 20 pmol of Na,K-ATPase was incubated for 30 min in the presence of 48 pmol PKA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 100 μM [γ-^{32}P]ATP at indicated concentrations of one of the detergents. Preincubation of duck salt gland Na,K-ATPase with 1 mM dithiothreitol for 30 min did not affect the enzyme activity.

results were obtained when the phosphorylation was preformed in the presence of 0.4% Chaps (data not shown).

The level of phosphorylation of the Na,K-ATPase α-subunit by the PKA depended on the incubation time and the concentration of PKA and Na,K-ATPase. The maximal level of phosphorylation was achieved within approximately 20-30 min in the presence of 48 pmol of PKA in the incubation medium (data not shown). The final values of the stoichiometry in the presence of 0.2% Triton X-100 were in the range 0.4-1.1 mol $^{32}P_i$ incorporated per mol α-subunit depending on the conditions.

The stoichiometry of the phosphorylation of Na,K-ATPase α-subunit as a function of α-subunit concentration at two different ATP concentration is presented in Fig. 2. It can be seen that at both ATP concentrations the highest level of $^{32}P_i$ incorporation was achieved at very low concentrations of the α-subunit (0.05-0.1 nM) and then decreased at higher concentrations.

Comparative effect of different detergents on the activity of Na,K-ATPase and the stoichiometry of its phosphorylation by PKA. To study the effect of Na,K-ATPase phosphorylation by PKA on enzyme activity *in vitro*, it is necessary to obtain phosphorylated enzyme with preserved activity. In order to find a suitable detergent, we compared the effects of different detergents on Na,K-ATPase activity and on the level of phosphorylation of the enzyme by PKA. Figure 3a shows the effect of four detergents on the activity of duck salt gland Na,K-ATPase: minimal loss of activity (about 20% at concentrations up to 0.4%) occurred with only two of these, *n*-octyl-β-glu-

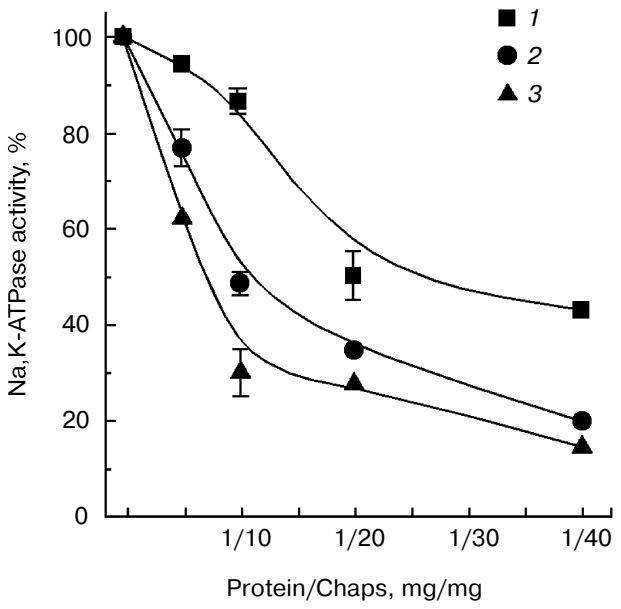

Fig. 4. Dependence of Na,K-ATPase activity on the protein/Chaps ratio in the incubation medium. Na,K-ATPase was preincubated for 10 min at different protein/detergent ratios and at Chaps concentrations of 0.3 (*1*), 0.4 (*2*), and 0.5% (*3*) as described in the legend to Fig. 3. Then the sample was transferred into incubation medium to measure the activity of Na,K-ATPase. The activity of the enzyme in the medium without Chaps is taken as 100%.

copyranoside and Chaps. The dependence of the stoichiometry of Na,K-ATPase phosphorylation on the concentration of these two detergents in the presence of 100 mM NaCl is shown in Fig. 3b. The highest level of $^{32}P_i$ incorporation into the α-subunit was observed in the presence of high concentrations of Chaps; thus, 0.26 and 0.37 mol P_i/mol α-subunit was incorporated in the presence of 0.4 and 0.5% Chaps, respectively, compared with 0.18 and 0.24 mol P_i/mol α-subunit with the same concentrations of *n*-octyl-β-glucopyranoside. Therefore, all subsequent experiments were performed using Chaps.

It was noted above (Fig. 2) that the stoichiometry of Na,K-ATPase phosphorylation by PKA decreased as enzyme concentration increased. The data presented in Figs. 3a and 3b were obtained at the same detergent concentrations but at different protein/detergent ratios because the optimal protein concentrations for measurement of Na,K-ATPase activity and its phosphorylation are different. Because the activity of the enzyme depends not only on the concentration of the detergent but also on protein/detergent ratio, we studied the change of Na,K-ATPase activity as a function of protein/detergent ratio using 0.3, 0.4, and 0.5% Chaps (Fig. 4). Based on this dependence, 0.4% Chaps concentration at protein/detergent ratios from 1 : 10 to 1 : 20 was chosen for further experiments. Under these conditions, 40-50% of the enzyme activity was retained and the stoichiometry of phosphorylation was within the range 0.18-0.28 mol P_i/mol of the α-subunit. Further increase in the protein/detergent ratio at constant Chaps concentration resulted in less inhibition of the Na,K-ATPase, but a simultaneous decrease in the stoichiometry of the phosphorylation. Conversely, decreasing the protein/detergent ratio increased the stoichiometry of the phosphorylation, but the inhibition of the Na,K-ATPase also increased.

Effects of monovalent cations and ATP on the stoichiometry of Na,K-ATPase phosphorylation. In an attempt to increase the level of PKA-mediated phosphorylation of Na,K-ATPase while maintaining constant Chaps concentration and without changing the protein/detergent ratio, the effect of different ligands on the stoichiometry of the phosphorylation was studied. It was reported recently by Feschenko and Sweadner [7] that the level of PKA-catalyzed phosphorylation of Na,K-ATPase from mammalian kidney in the presence of Triton X-100 depended on the conformation of the enzyme. The highest phosphorylation was achieved in the presence of Triton X-100 without any additions. A lower level of phosphorylation was obtained in the presence of Na^+, and the lowest level in the presence of K^+ [7]. In the medium with Triton X-100, the results obtained in our experiments (Fig. 5a) were similar to those described by Feschenko and Sweadner [7]. Surprisingly, however, when Chaps was the detergent, very different results were found (Fig. 5b). Incorporation of ^{32}P into the enzyme was maximal in medium with K^+ and minimal in medium with Na^+. In medium without ligands the stoichiometry of Na,K-ATPase phosphorylation was intermediate (Fig. 5b). This suggests that Chaps affects the conformation of

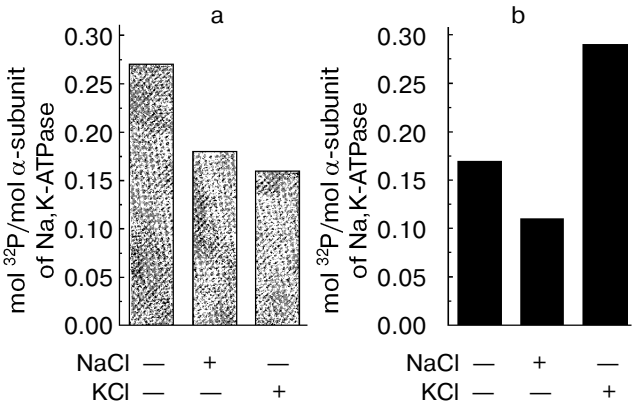

Fig. 5. Effects of Na^+ and K^+ on the stoichiometry of Na,K-ATPase phosphorylation by PKA in medium containing 0.2% Triton X-100 (a) and 0.4% Chaps (b). Na,K-ATPase (20 pmol) was incubated with 48 pmol PKA for 35 min in the presence of 50 μM [γ-^{32}P]ATP and 100 mM NaCl or KCl under conditions described in "Materials and Methods".

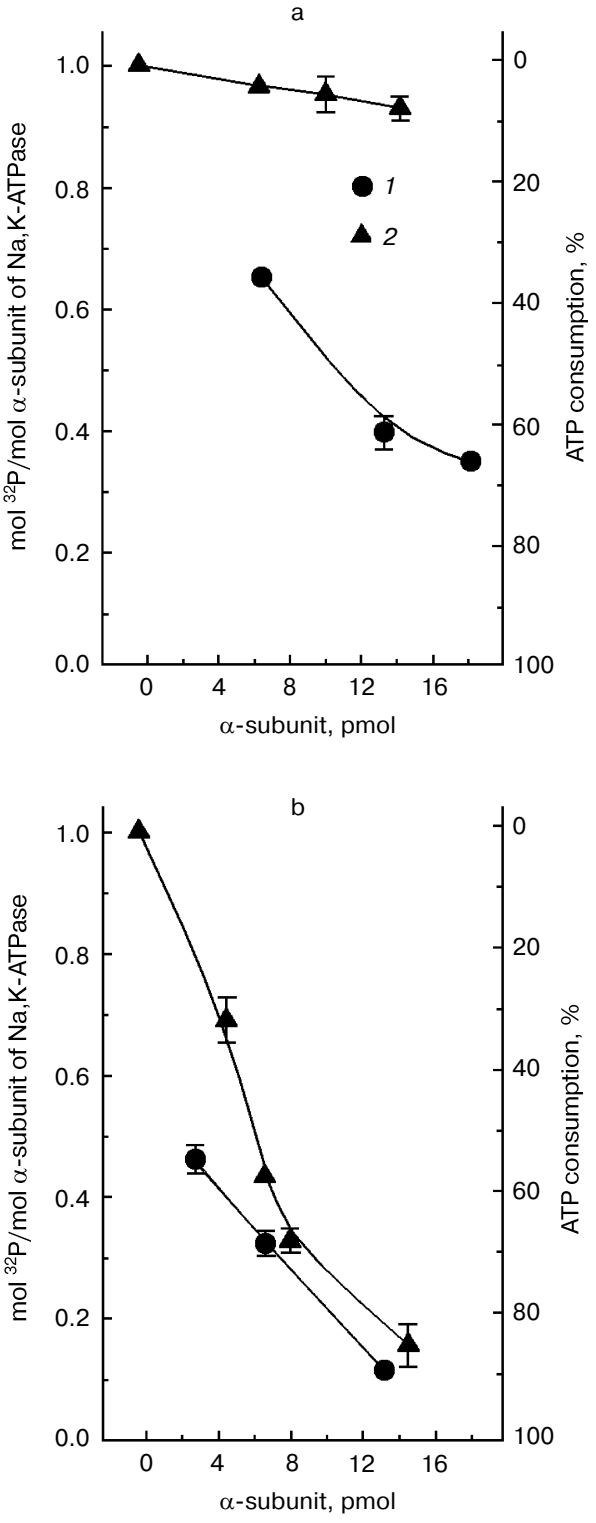

Fig. 6. Change of the stoichiometry of duck salt gland Na,K-ATPase (10 pmol) phosphorylation (*1*) and ATP concentration in the incubation medium (*2*) after 30 min of incubation in the presence of 48 pmol PKA depending on concentration of Na,K-ATPase α-subunit in the presence of 0.4% Chaps and 100 mM KCl (a) or 100 mM NaCl (b). Phosphorylation was done in medium containing 100 μM [γ-^{32}P]ATP under conditions described in "Materials and Methods".

Na,K-ATPase in a different way than Triton X-100. Therefore, in the presence of Chaps the effects of Na^+ and K^+ on the accessibility of phosphorylation site to PKA will be different than in the presence of Triton X-100. However, there is also another explanation of these data.

Since in the medium with Chaps Na,K-ATPase is active and in the medium with Triton X-100 the enzyme is completely inactivated, we suggested that in the presence of Chaps Na,K-ATPase being activated by K^+ (which may be present in the medium as impurity) consumes ATP during the incubation time. Since ATP is also a substrate for PKA, the decrease of nucleotide concentration results in the decrease of PKA activity and the level of Na,K-ATPase phosphorylation. It can be seen from Fig. 6a that during the incubation of Na,K-ATPase with 100 mM KCl the level of ATP in the incubation medium decreased only slightly (by about 10% during the 30-min incubation with 10 pmol of Na,K-ATPase α-subunit). The final stoichiometry of the phosphorylation under these conditions was close to 0.4 mol P_i/mol α-subunit. However, in the presence of 100 mM NaCl the final ATP concentration was only 20% of the initial value after the 30-min incubation with the same amount of Na,K-ATPase α-subunit (Fig. 6b). The stoichiometry of the phosphorylation in this case was about 0.1 mol P_i/mol of α-subunit.

In our experiments and those in the study of Feschenko and Sweadner [7], the highest level of Na,K-ATPase phosphorylation in the presence of Triton X-100 was obtained in medium without additions. The simplest explanation for this result may be that ionic strength inhibits PKA activity itself, as has been reported previously [20]. The data presented in the table support this suggestion: addition of 100 mM KCl as well as 100 mM NaCl significantly decreased PKA activity when histone H1 was being phosphorylated.

We have noted that an increase in ^{32}P incorporation into the α-subunit of Na,K-ATPase was also observed when ATP concentration was increased (Fig. 2). This appears to be due to the increase of PKA activity because the same effect was observed in the presence of Triton X-100. To investigate this further, we compared the effect of different ATP concentrations on the stoichiometry of Na,K-ATPase phosphorylation and activity of PKA with histone H1 as the substrate (Fig. 7). The data show that the level of Na,K-ATPase phosphorylation correlates with PKA activity quite well. Increasing the ATP concentration from 20 to 300 μM resulted in about 1.5-fold increase of both parameters. As can be seen from Fig. 7, increasing the ATP concentration higher than 300 μM does not result in a significant increase of the level of $^{32}P_i$ incorporation. Beside that, to preserve the specific radioactivity of [γ-^{32}P]ATP when the total ATP concentration is increased, it is necessary to increase proportionally the total amount of radioactive ATP. For these rea-

Effect of monovalent cations on the activity of PKA. The catalytic subunit of PKA (48 pmol) was incubated with histone H1 (1 mg/ml) in the presence of 0.2% Triton X-100 under conditions described in "Materials and Methods"

Cation concentration, mM		PKA activity, %
KCl	NaCl	
0	0	100
100	0	43.7 ± 7.1
0	100	41.5 ± 5.4

sons, we chose 300 μM ATP concentration for further experiments.

Detection of phosphoamino acids phosphorylated by PKA in the α-subunit of Na,K-ATPase. To identify amino acids phosphorylated by PKA in our experiments, purified duck salt gland Na,K-ATPase was phosphorylated by PKA in the presence of 0.2% Triton X-100 and 0.4% Chaps. When the stoichiometry of phosphorylation of the α-subunit in the presence of 0.2% Triton X-100 was 1.1 mol of P_i/mol α-subunit, two phosphorylated amino acids, phosphoserine and phosphothreonine, were identified. The content of phosphothreonine in the phosphorylated α-subunit compared to phosphoserine was low and did not exceed 10% of the total content of phosphoamino acids. When phosphorylation was carried out in the presence of 0.4% Chaps and maximal stoichiometry was about 0.6 mol of P_i/mol α-subunit, only phosphoserine was detected (data not shown).

Effect of PKA-mediated phosphorylation on Na,K-ATPase activity. Our data demonstrate that in medium containing Chaps (0.4%, the protein/detergent ratio being 1 : 10), the activity of Na,K-ATPase decreased by 40-50%. It is possible to phosphorylate the Na,K-ATPase by PKA with quite high stoichiometry under these conditions in the presence of 100 mM KCl and 300 μM ATP. We carried out incubation of Na,K-ATPase with PKA in the presence of Chaps, 100 mM KCl, and 300 μM ATP. At different time intervals, aliquots were removed for simultaneous measurement of the Na,K-ATPase activity and the phosphorylation stoichiometry. The results of these experiments are presented in Fig. 8. It can be seen that the level of Na,K-ATPase phosphorylation increased with time between 5-35 min of incubation, from 0.2 to 0.5 mol P_i/mol of Na,K-ATPase α-subunit (Fig. 8a, curve *2*). In the same incubations, the activity of Na,K-ATPase decreased over the same time interval, falling to about 36% of the initial value (Fig. 8a, curve *3*). In control incubations with PKA and without ATP added, however, the enzyme activity remained much higher, with about 80% of the original activity remaining after 35 min (Fig. 8a, curve *1*). Comparing enzyme activities after 35 min, the activity in the presence of PKA and ATP was only about 45% of the value without PKA. Hence, a correlation between incorporation of P_i into the α-subunit of Na,K-ATPase and the loss of its enzyme activity was observed (Fig. 8b).

DISCUSSION

Regulation of Na,K-ATPase activity via its phosphorylation by various protein kinases is thought to play an important role in regulation of electrolyte balance of animal cells [21]. Despite the fact that the α-subunit of Na,K-ATPase purified from various tissues has been shown to be a direct target for PKA both *in vitro* [6-12] and in living cells [9, 12, 22], there is no definitive answer concerning the effect of protein kinase-mediated phosphorylation on the activity of the enzyme.

Technical problems have impeded progress because the phosphorylation site of the enzyme is inaccessible for PKA both in the preparations of purified Na,K-ATPase [7, 9-11] and in the vesicles of the microsomal fraction [9]. Therefore, phosphorylation occurs only after treatment of preparations by detergents. This fact initially led to doubts about the physiological importance of PKA-mediated phosphorylation of Na,K-ATPase. However, it has been

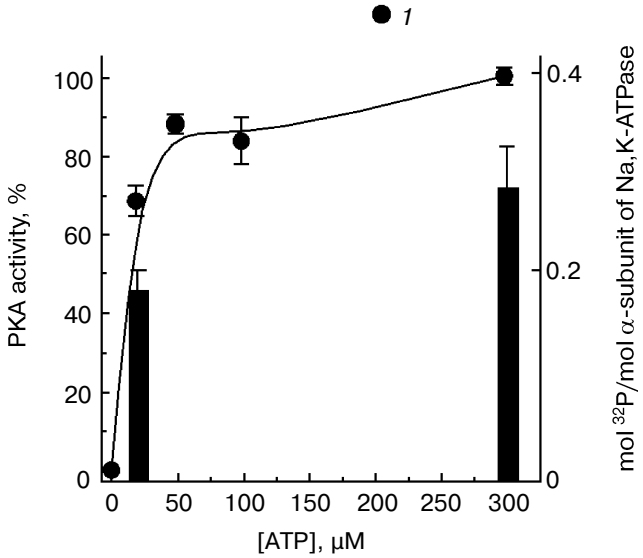

Fig. 7. Dependence of PKA activity (*1*) and the stoichiometry of duck salt gland Na,K-ATPase phosphorylation by PKA (vertical bars) on the ATP concentration. Na,K-ATPase (20 pmol) was incubated with PKA (48 pmol) in the presence of 0.2% Triton X-100. Other experimental details are described in "Materials and Methods".

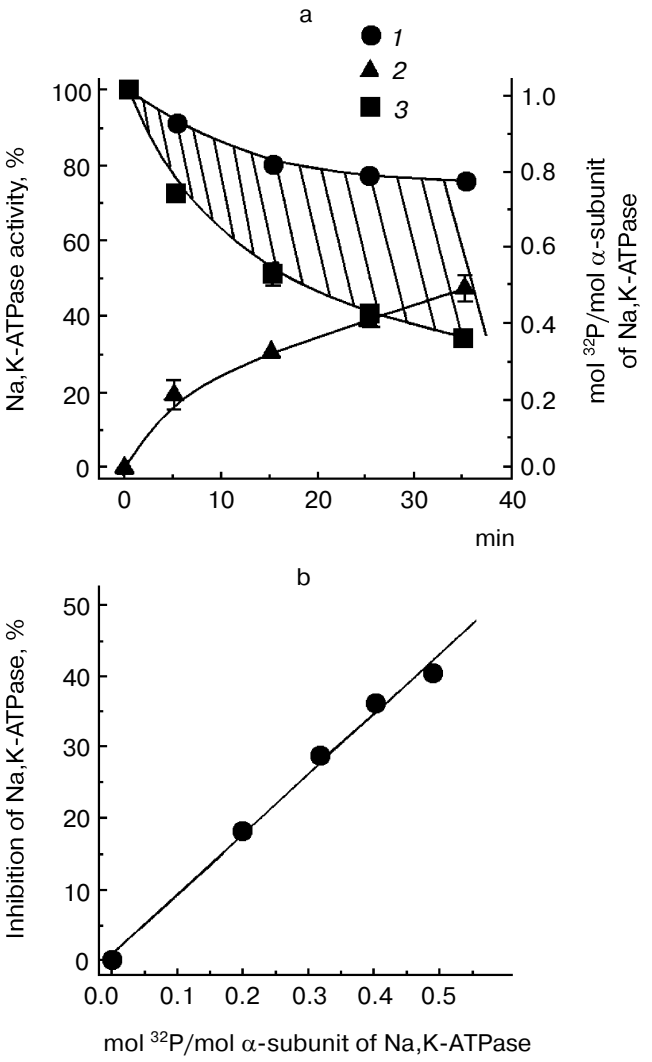

Fig. 8. a) Effect of duck salt gland Na,K-ATPase phosphorylation by PKA on the enzyme activity. Na,K-ATPase (200 pmol) was incubated with PKA (480 pmol) in the presence of 0.4% Chaps, 100 mM KCl, and 300 μM [γ-^{32}P]ATP in a volume 0.6 ml. Other details of the experiment are described in "Materials and Methods". Aliquots with a volume 60 μl were taken off at the indicated times after the start of the reaction for the measurement of Na,K-ATPase activity (*1*) and the stoichiometry of the phosphorylation (*2*). Control samples were incubated under the same conditions but without ATP (*3*). The shaded region shows activity that is inhibited as result of the phosphorylation of the Na,K-ATPase. b) Correlation between the incorporation of $^{32}P_i$ into the Na,K-ATPase α-subunit and inhibition of the enzyme activity.

shown that phosphorylation of Na,K-ATPase by PKA takes place in intact cells in response to stimulation by endogenous PKA, and that this phosphorylation results in a change of Na,K-ATPase activity. First, there are data of Fisone et al. showing that the process of activation of PKA in COS cells leads to the inhibition of wild-type Na,K-ATPase from rat kidney expressed in these cells and does not affect the activity of the mutant Na,K-ATPase in which Ser-943 was substituted by alanine [13]. Also, Blanko et al. showed that activation of PKA in Sf-9 insect cells resulted in the inhibition of α1β1- and α2β1-isoenzymes and in the activation of α3β1-isoenzyme of Na,K-ATPase expressed in these cells [22]. In these experiments, PKA activation did not influence the rate of Na,K-ATPase endocytosis, thus Na,K-ATPase inhibition was the result of the change of molecular activity of the enzyme. Taking into account all these data, we suggest that activation of PKA in living cells results in the inhibition of α1β1-isoenzyme of Na,K-ATPase, and this inhibition is connected with the phosphorylation of the only serine residue of the enzyme α-subunit corresponding to Ser-943 of Na,K-ATPase α-subunit from rat kidney.

A possible explanation for the discrepancies between the accessibility of the Na,K-ATPase phosphorylation site *in vitro* and in the intact cell is the following. The living cell contains proteins and/or other cell constituents which, being bound to Na,K-ATPase, induce a change in conformation of the enzyme and, in this way, alters the accessibility of the phosphorylation site to PKA. This component (or components) was perhaps present in the rabbit kidney preparation of Na,K-ATPase which was used in the study of Mardh [6] because Na,K-ATPase in these experiments was successfully phosphorylated by PKA in the absence of the detergent.

We mentioned above that Cornelius and Logvinenko reconstituted Na,K-ATPase from pig kidney (α1β1-isoenzyme) and shark rectal glands (α3-isoform according to immunological test) into phospholipid vesicles and have performed phosphorylation of these enzymes by PKA without the addition of detergents [8]. Under these conditions, phosphorylation resulted in the activation of transport provided by Na,K-ATPase from rectal glands and did not influence on the transport of cations by Na,K-ATPase from pig kidney [8]. Therefore, the phosphorylation of purified α1β1-isoenzyme of Na,K-ATPase does not lead to its inhibition *in vitro*, but it inhibits this isoenzyme in living cells. This means that either phosphorylation of Na,K-ATPase is not enough to provide its inhibition or the inhibition is not the result of phosphorylation.

This conclusion led us to more precise analysis of studies where authors did not find the effect of phosphorylation on the activity of α1β1-isoenzyme of Na,K-ATPase, in particular, the paper published by Cornelius and Logvinenko [8]. We should noted that although these authors indicate significant stoichiometry of phosphorylation of Na,K-ATPase from pig kidney (0.4-1.15 mol P_i/mol α-subunit), they unfortunately did not show in the paper the values of stoichiometry for the experiments where phosphorylation did not affect the enzyme activity. In addition, solubilization of Na,K-ATPase before its reconstitution into vesicles resulted in a significant loss of enzyme activity (for Na,K-ATPase from pig kidney, about 4-5-fold) [8]. Thus, we tried to perform the phosphoryla-

tion of Na,K-ATPase by PKA under conditions when about 50% of the activity is preserved and significant stoichiometry of the phosphorylation is achieved.

Of three nonionic detergents (*n*-octyl-β-glucopyranoside, Brij 35, Triton X-100) and one zwitterionic detergent (Chaps) tested in our study, only *n*-octyl-β-glucopyranoside and Chaps caused a relatively small decrease of enzyme activity at concentrations 0.2-0.4% (Fig. 3a), and the effect of Chaps at concentrations of 0.4-0.5% on the Na,K-ATPase activity was minimal. However, the effect of any detergent on an integral membrane protein depends on both detergent concentration and protein/detergent ratio. In the presence of 0.5% Chaps, even a protein/Chaps ratio of 1 : 5 resulted in the loss of Na,K-ATPase activity, which fell to approximately 60% of its original value (Fig. 4). Another complication is the dependence of the phosphorylation on the Na,K-ATPase concentration (decrease of ^{32}P incorporation with increasing Na,K-ATPase concentration, Fig. 2). This kind of dependence may be connected with the occurrence of substrate inhibition of PKA by Na,K-ATPase. Apparent substrate inhibition of protein kinase C activity by Na,K-ATPase was described earlier [23]. The authors suggested that this could be characteristic of the interaction between protein kinase and a membrane-embedded substrate. Therefore, the combination of three factors (dependence of phosphorylation on Na,K-ATPase concentration, dependence of Na,K-ATPase activity on protein/detergent ratio and detergent concentration, dependence of phosphorylation on detergent concentration) creates a complex problem when trying to find conditions that provide a high level of Na,K-ATPase phosphorylation while minimizing the loss of activity even in the presence of Chaps, which provides minimal inhibition of Na,K-ATPase.

To minimize this problem, we studied the effect of different ligands on the level of Na,K-ATPase phosphorylation by PKA. It was shown earlier that the level of enzyme phosphorylation by PKA depends on whether Na^+ or K^+ is present in the incubation medium [7]. These data led the authors to suggest that the accessibility to PKA of the phosphorylation site(s) on the Na,K-ATPase depend on the enzyme conformation. We have found that in a medium containing Chaps that did not inhibit the Na,K-ATPase completely, the effect of Na^+ and K^+ on the stoichiometry of the phosphorylation is opposite to that observed in a medium with Triton X-100 (Fig. 5). ATP is known to be a common substrate for PKA and Na,K-ATPase, and Na,K-ATPase needs Na^+ and K^+ for the hydrolysis of ATP. However, the enzyme can also hydrolyze ATP in a medium without K^+ (so-called Na-ATPase) [24]. On the other hand, the sensitivity of the enzyme to K^+ is quite high ($K_{0.5}$ for K^+ is 0.1-0.2 mM versus 1.5-2 mM for Na^+) [25]. Therefore, K^+ that is present in reagents as impurities may have activated Na,K-ATPase. Considering this, we directly measured ATP exhaustion during enzyme incubation with PKA in the presence of Chaps and Triton X-100 (Fig. 6). From these data we concluded that in the medium with Chaps the lower level of phosphorylation of Na,K-ATPase by PKA may be due to the consumption of ATP during incubation by active Na,K-ATPase.

Our results also show that the higher level of phosphorylation of Na,K-ATPase α-subunit in Triton-containing medium where Na,K-ATPase is not active may be achieved in a medium without additions. We found that there is inhibition of PKA (as measured with histone H1 substrate) in a medium with 100 mM KCl or NaCl compared with a medium without cations (table). Significant inhibition of PKA by 120 mM NaCl was shown by Mardh [6], who measured PKA activity with a synthetic nanopeptide that represents part of the phosphorylation site of the L-type of pyruvate kinase. On the other hand, Feschenko and Sweadner did not find any significant effect of 140 mM NaCl or 100 mM KCl on the activity of PKA with kemptide as the substrate [7]. This suggests that the effect of monovalent cations on PKA activity depends on the structure of the PKA substrate used, and ionic strength inhibits PKA when it phosphorylates histone H1, Na,K-ATPase, and Mardh's synthetic nanopeptide.

We also found that an increase in ATP concentration up to 300 μM results in an increase of stoichiometry of Na,K-ATPase phosphorylation by PKA in the medium containing Chaps and K^+ where hydrolysis of ATP is negligible (Fig. 8). Although the K_m of PKA for ATP is about 5 μM [20], it is known that an increase in substrate concentration within the range higher than the K_m value up to 20-fold should also result in an increase of enzyme activity. It is also known that ATP affects Na,K-ATPase conformation, being bound in a low affinity site with K_m about 100 μM [25]. However, the possibility of conformational change induced by ATP in the presence of Triton X-100 seems unlikely.

Under conditions with KCl, 0.4% Chaps, and relatively high ATP concentration (300 μM), we were able to phosphorylate active Na,K-ATPase with a sufficiently high stoichiometry. It should be noted that determination of the precise stoichiometry of phosphorylation for Na,K-ATPase is a difficult problem. A main difficulty is the method of protein determination. It has been shown that the Lowry method is more reliable to measure Na,K-ATPase concentration [26]. The use of other methods may result in overestimation of the stoichiometry of phosphorylation. Because we used in our experiments the Lowry method to measure Na,K-ATPase concentration, the stoichiometry of the phosphorylation in our experiments should be estimated with sufficient accuracy.

To demonstrate the direct effect of PKA-mediated phosphorylation on Na,K-ATPase activity, we phosphorylated Na,K-ATPase for different time intervals. This approach gave us the possibility of measuring an increase in ^{32}P incorporation into Na,K-ATPase α-subunit and the corresponding level of enzyme activity at each level of the phosphory-

lation. It is important to note that aliquots for measurement of these two parameters were taken from the same sample, this significantly decreasing the error. Our data clearly demonstrated that increasing stoichiometry of phosphorylation correlates with the loss of Na,K-ATPase activity (Fig. 8).

In the present study we confirmed results of Chibalin et al. that two amino acids (serine and threonine) are phosphorylated by PKA in Na,K-ATPase α-subunit from duck salt glands in the presence of Triton X-100 [9]. However, the amount of phosphothreonine was very small. Also, in the medium with Chaps only phosphoserine was detected after PKA-mediated phosphorylation of the enzyme. Although the sequence of duck salt gland Na,K-ATPase α-subunit is still unknown, it has been shown from immunological data [27, 28] and also N-terminal sequencing of α- and β-subunits [28, 29] as well as kinetic behavior [27] that the enzyme belongs to the family of α1β1-isozymes. According to a hypothetical model of the membrane folding of Na,K-ATPase α-subunit with 10 transmembrane segments, the PKA phosphorylation site (Ser-943) is in the short cytoplasmic loop located between two putative membrane-spanning segments in the C-terminal part of the molecule [13]. For mammalian and amphibian kidney Na,K-ATPase this site was shown to be the only one which is phosphorylated by PKA. Because Ser-943 is located in a highly conservative sequence present in all isoforms of the α-subunit, this suggests that a corresponding serine residue may be phosphorylated in the α-subunit of duck salt gland Na,K-ATPase.

Therefore, we conclude that the phosphorylation of serine residue(s) of purified duck salt glands Na,K-ATPase α-subunit results in progressive inhibition of the Na,K-ATPase. There is a correlation between the phosphorylation of the α-subunit and the loss of enzyme activity.

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