Identification of the Phosphorylation Site for cAMP-dependent Protein Kinase on Na⁺,K⁺-ATPase and Effects of Site-directed Mutagenesis*

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Phosphorylation of purified Na⁺,K⁺-ATPase by cAMP-dependent protein kinase (protein kinase A) decreases the activity of this enzyme. We have now shown, using several experimental approaches, that a highly conserved seryl residue on the catalytic (α) subunit of Na⁺,K⁺-ATPase, corresponding to Ser⁹⁴⁸ of the rat a1 isoform, is the phosphorylation site for protein kinase A. cDNAs corresponding to wild-type Na⁺,K⁺-ATPase and Na⁺,K⁺-ATPase in which Ser⁹⁴⁸ was mutated to Ala were transfected into COS cells. Treatment of the transfected cells with forskolin plus 3-isobutyl-1-methylxanthine resulted in a decrease in the activity of the wild-type enzyme but not in that of the mutated enzyme. The results suggest that, in intact cells, the activity of the Na⁺,K⁺-ATPase is regulated in part by signal transduction pathways that use protein kinase A-dependent phosphorylation of the Na⁺,K⁺-ATPase α subunit.

Na⁺,K⁺-ATPase is a ubiquitous enzyme essential for the maintenance of electrolyte balance in virtually all tissues. Na⁺,K⁺-ATPase is particularly abundant in kidney and brain. In kidney, the enzyme plays a key role in regulating sodium reabsorption. In the brain, it is responsible for the maintenance of the ionic gradients of Na⁺ and K⁺ that underlie the resting and action potentials. In these tissues, a large portion of cellular energy is expended by this enzyme.

Previous work has shown that the activity of Na⁺,K⁺-ATPase in renal tubule cells and neurons is modulated by a number of hormones and neurotransmitters (1–5) A possible common mechanism by which these substances affect the activity of Na⁺,K⁺-ATPase is by regulating the state of phosphorylation of its catalytic (α) subunit (6–8). Experiments in vitro showed that cAMP-dependent protein kinase (protein kinase A)¹ and protein kinase C can phosphorylate the α subunit of purified Na⁺,K⁺-ATPase (9–11), thereby inhibiting Na⁺,K⁺-ATPase activity (10). Protein kinase A phosphorylates the α subunit at a single seryl residue (10). We now report the identification of the seryl residue of the a1 subunit of rat Na⁺,K⁺-ATPase, which is phosphorylated by protein kinase A, and provide evidence for the physiological importance of this phosphorylation site in intact cells.

EXPERIMENTAL PROCEDURES

Sequence Analysis of Peptide Fragments—Purified Na⁺,K⁺-ATPase from rat renal cortex, which contains only the α1 isoform, was phosphorylated as described below. Analytical SDS-polyacrylamide gel electrophoresis (PAGE) with subsequent autoradiography was performed to measure the stoichiometry of phosphorylation. The α subunit was subjected to preparative SDS-PAGE and cut from the gel after detection by negative staining with 1 M KCl as described (12), except that acetic acid was omitted to avoid fixation (13). The remaining part of the gel was stained with Coomassie Brilliant Blue to confirm the absence of the Na⁺,K⁺-ATPase 100-kDa α subunit. Electrophoresis of the excised gel pieces was carried out in 10 mM NH₄HCO₃, pH 9 (13). The total radioactivity of the eluate was 60,000 cpm, corresponding to 4 pmol (0.4 μg) of protein. This material was digested in two different batches with roughly equimolar amounts of endoprotease Lys-C in 0.5 M NH₄HCO₃, pH 8.1, for 4 h at 37 °C. The digests were subjected to automated Edman degradation (MilliGen Prosequencer 6600), and the radioactivity eluted after each cycle was determined by scintillation counting (LKB 1211 Rackbeta), after fractionation of the elutes from the on-line HPLC to allow separate collection of four fractions/cycle. These fractions corresponded to the eluates at 1–3 min (prevoid volume), 3–5 min (acidic material eluting before PTH-Leu), 6–10 min (PTH-Asp through PTH-Leu). The reaction mixture was 100 μg/ml of Na⁺,K⁺-ATPase α subunit. The sequence of the peptide was NH₂-ICKTRRNSFPQG-CSO-H. This sequence corresponds to residues 936–948 of the α1 isoform of rat Na⁺,K⁺-ATPase and is conserved in the different isoforms of Na⁺,K⁺-ATPase α subunit, including the α3 isoform present in the shark rectal gland. The peptide was synthesized by the Rockefeller University Protein Sequencing/Biopolymers Facility, and purified by preparative reversed-phase HPLC. It was >95% pure as analyzed by HPLC and had the expected amino acid composition and mass spectrum (data not shown).

Phosphorylation Assays—Phosphorylation of purified rat kidney and shark rectal gland Na⁺,K⁺-ATPase (14, 15) was carried out at 22 °C in a reaction volume of 100 μl containing 50 mM Hepes pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 0.1% Tyrode X-100, and 2 μg of Na⁺,K⁺-ATPase. The final concentration of protein kinase A catalytic subunit in the reaction mixture was 100 μg/ml. Reactions were initiated by the addition of 100 μM [γ-³²P]ATP (5 × 10⁶ cpm/nmol) and stopped after 30 min.

¹ 3-isobutyl-1-methylxanthine; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; bp, base pair(s).

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For sequence analysis of peptide fragments, purified Na⁺,K⁺-ATPase from rat renal cortex was phosphorylated under the same conditions, except that the reaction was carried out for 60 min, in the presence of 1 mM \( \gamma^-32P\)ATP. The reactions were terminated by the addition of SDS-containing stop solution (final concentrations, 0.5% SDS, 40 mM Tris-HCl, pH 6.8, 8% acrylamide, 1% EDTA, 0.2% β-mercaptoethanol, 0.01% sodium borohydride). Samples were subjected to SDS-PAGE according to the method of Laemmli (16), using prestained molecular weight standards.

Phosphorylation of the synthetic peptide was performed as described for the Na⁺,K⁺-ATPase, except that 100 μM peptide, 1 mM \( \gamma^-32P\)ATP (3 x 10⁶ cpm/μl) from 190 μg calmodulin subunit of protein kinase A were used. Reactions were stopped after 30 min by the addition of glacial acetic acid (final concentration, 10%), and aliquots were spotted on phosphocellulose filters (P81, Whatman). Filters were washed in distilled water for 20 min, and the incorporation of \( \gamma^-32P\) into peptide was determined by Cerenkov counting. Preparative phosphorylation of the synthetic peptide was performed with protein kinase A catalytic subunit (0.5 μg/μl), in the presence of 1 mM unlabeled ATP and 1 mM dithiothreitol (Sigma).

For kinetic analysis of peptide phosphorylation, the peptide was incubated for 5 min at 30°C in a reaction volume of 100 μl containing (final concentrations): 50 mM HEPES (pH 7.5), 0.2 mg/ml bovine serum albumin, 100 μM \( \gamma^-32P\)ATP (2 x 10⁴ cpm/μl), 0.5 μg/ml catalytic subunit of protein kinase A were used. Reactions were stopped after 30 min by the addition of glacial acetic acid (final concentration, 10%), and aliquots were spotted on phosphocellulose filters (P81, Whatman). Filters were washed in distilled water for 20 min, and the incorporation of \( \gamma^-32P\) into peptide was determined by Cerenkov counting. Preparative phosphorylation of the synthetic peptide was performed with protein kinase A catalytic subunit (0.5 μg/μl), in the presence of 1 mM unlabeled ATP and 1 mM dithiothreitol (Sigma).

**Two-dimensional Phosphopeptide Mapping—** Pieces containing \( \gamma^-32P\)-labeled peptide a subunit from shark rectal gland and from rat renal cortex were excised from dried gels, washed with two changes of 10% acetic acid/30% methanol, three changes of 50% methanol, and lyophilized. For characterization of the \( \gamma^-32P\)-labeled peptide, the phosphopeptide was separated from unincorporated \( \gamma^-32P\)ATP by chromatography over a 1-ml column of Dowex AG 1-X8 (acetate form, 200-400 mesh) equilibrated in 30% acetic acid and then lyophilized. One ml of 50 mM NH₄HCO₃, pH 8.0, containing threonine (Calbiochem; 50 μg/ml), was added to the dried gel pieces or recovered peptide, and the mixture was incubated at 37°C for 20 h. The supernatants were removed, and the gel pieces were washed with an additional 0.5 ml of 50 mM NH₄HCO₃ at 37°C for 4 h. The collected supernatants were lyophilized. The lyophilized residues were resuspended in electrophoresis buffer (10% acetic acid, 1% pyridine, pH 3.5) and spotted 10 cm from the right and 4 cm from the bottom on thin-layer cellulose sheets (20 x 20 cm, Eastman). Phosphopeptides were separated by electrophoresis at 400 V for 60 min in the first dimension (left, positive), followed by chromatography in the second dimension in a buffer containing pyridine:1-butanol:water:acetic acid (10:15:12:3, v/v). Dried spots were subjected to autoradiography.

**Preparation of Antibodies—** The production of phospho-specific antibodies followed the general procedures previously described (17). Synthetic peptide phosphorylated by protein kinase A was separated from unreacted ATP by chromatography over a 1-ml column of Dowex AG 1-X8 (acetate form, 200-400 mesh) equilibrated in 30% acetic acid and then lyophilized. The phosphorylated peptide was removed by the addition of glacial acetic acid (final concentration, 10%), and aliquots were spotted on phosphocellulose filters (P81, Whatman). Filters were washed in distilled water for 20 min, and the incorporation of \( \gamma^-32P\) into peptide was determined by Cerenkov counting. Preparative phosphorylation of the synthetic peptide was performed with protein kinase A catalytic subunit (0.5 μg/μl), in the presence of 1 mM unlabeled ATP and 1 mM dithiothreitol (Sigma).

**Expression Vector Constructions—** The mutagenized or wild-type insert was digested from pBSKS(+)Al and subcloned into the pSH site of the polylinker region of vector pXM (a gift from the late Professor Håkan Persson, Department of Medical Chemistry, Karolinska Institute). pXM is a highly evolved eukaryotic expression vector containing eukaryotic regulatory elements (18). Three constructs were generated: construct pXM-Al(+) contained wild-type a1 cDNA insert in the sense orientation, construct pXM-Al(A943) but in the antisense orientation; construct pXM-Al(Al943) contained the mutated insert in the sense orientation.

**Cell Culture and DNA Transfection—** COS cells (a monkey kidney cell line), a gift from the late Professor Håkan Persson, were grown at 37°C, 5% CO₂ and 95% humidified air, on 90-mm culture dishes in complete Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Media were changed every 3 days. For transfection, the vectors were linearized with NotI, and the COS cells were transfected using Chen and Okayama's (19) modified calcium-phosphate/DNA precipitation. Briefly, a 30-50 μg competent dish of COS cells was initially incubated for 4 h at 37°C with an appropriate amount of calcium-phosphate/DNA precipitate, containing 25 μg of the recombinant expression vectors or pXM alone, followed by 3 min of 15% glycerol treatment. After a brief wash with 10 ml of serum-free DMEM, the cells were incubated in complete DMEM, and the plates were allowed to grow to confluence.

**Transfected Selection and Colony Isolation—** Due to a difference in ouabain binding affinity between monkey and rat α1 subunits, rat cells survive concentrations of ouabain that kill monkey cells (COS cells). To increase our ability to select transfected cells, in which the cDNA encoding the rat α1 subunit had been transfected, expressed in a stable way, and assembled with the monkey β subunit to form a functional Na⁺,K⁺-ATPase in the plasma membrane. Following DNA transfection, confluent plates were split 1:5, and 1:20 into confluent plates. 7 days of incubation, the media were replaced with media containing 10 μg/ml phototoxin to select for transfected monkey β subunit. The expression of ouabain concentration was maintained by changing the medium every other day. Seven to ten days later, many colonies were visualized in pXM.
Determination of Na+,K+-ATPase Activity—Confluent dishes were preincubated for 20 min in DMEM in the absence or presence of 10 μM forskolin (Sigma) plus 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) under standard culture conditions as described above, except that fetal calf serum was not present in the media. In some experiments, 0.1 mM H89 (Calbiochem), a selective protein kinase A inhibitor (20), was also added to the preincubation media. Plasma membranes were prepared at 4 °C (21), washed once with 1 mM Tris-HCl, pH 7.5, and resuspended in a buffer containing 75 mM Tris-HCl, pH 7.5, 12.5 mM MgCl₂, 1.5 mM EDTA. Crude plasma membranes were quickly frozen on dry ice and thawed at room temperature to lyse vesicles formed during the membrane preparation. Aliquots of membrane fragments were incubated for 15 min at 37 °C in 100 μl of a solution containing 20 mM KCl, 5 mM MgCl₂, 30 mM Tris-HCl, pH 7.4, 1 mM EGTA, 3 mM Tris-ATP, and [γ-32P]ATP (specific activity, 10 Ci/mmol). To prevent dephosphorylation of Na+,K+-ATPase, the buffer was supplemented with the protein phosphatase inhibitors okadaic acid (250 μM) and FK506 (25 μM). NaCl was added in concentrations varying from 0 to 70 mM. To maintain a constant osmolarity, choline chloride was also added, so that the sum of NaCl and choline chloride was kept constant at 130 mM. All studies were performed in the presence of 10 μM ouabain, to inhibit endogenous COS cell Na+,K+-ATPase activity. An amount of enzyme was selected so that total ATP hydrolysis did not exceed 20%, and ATP hydrolysis was linear with time. The reaction was stopped by the addition of 700 μl of activated charcoal. The 32P liberated was determined in the supernatant after centrifugation. For the determination of ouabain-insensitive rat ATPase activity, NaCl and KCl were omitted, and 5 mM ouabain was added. Protein content in cell membranes was determined by the method of Bradford (22), using a kit from Bio-Rad. Kinetic analysis was carried out as described (23).

RESULTS

Radiosequence Analysis—The in vitro phosphorylated, 32P-labeled α subunit of Na+,K+-ATPase from rat kidney was isolated by SDS-PAGE and electroelution and digested with Lys-C endoproteinase, and the mixture was used for direct radiosequence analysis. A significant increase in radioactivity was detected in the fifth cycle from that fraction of the on-line HPLC eluate containing highly acidic material (3–6 min eluate). The pattern was reproduced in two independent experiments. A comparison of this pattern with the Lys/Ser distribution in the protein (26) shows that Ser217, Ser499, and Ser943 are the only seryl residues situated 5 residues after a lysyl residue. Hence, it is concluded that one of these seryl residues is the one phosphorylated in the intact subunit. The residues adjacent to Ser943, but not those adjacent to Ser217 or Ser499, confirm to the expected consensus site for protein kinase A-dependent phosphorylation.

Phosphorylation of Synthetic Peptide and Phosphoamino Acid Analysis—The α subunit synthetic peptide (296–448) (IKC-TRRNSVFQGG), corresponding to the putative phosphorylation site on the Na+,K+-ATPase, was a very good substrate for protein kinase A and was phosphorylated in the presence of [γ-32P]ATP to a stoichiometry of ~1 mol/mol. Kinetic analysis of the phosphorylation of the peptide by protein kinase A yielded a Kₘ value of 12 μM and a Vₘₐₓ value of 4.7 μmol/min/mg. Phosphoamino acid analysis revealed that radioactivity was associated only with phosphorylation of seryl residues. This indicated that Ser943 and not Thr939 was the phosphorylation site (Fig. 1).

Thermolytic Phosphopeptide Mapping—Purified Na+,K+-ATPase α subunit from shark rectal gland and from rat renal cortex and the synthetic peptide were subjected to phosphorylation with protein kinase A in the presence of [γ-32P]ATP and digested with thermolysin, after which two-dimensional phosphopeptide mapping was performed. A radioactive phosphopeptide (peptide 1) obtained from the thermolytic digestion of the shark rectal gland phosphorylated α subunit (Fig. 2A) and of the synthetic peptide (Fig. 2B) was found to comigrate (Fig. 2C). Idential results were obtained upon digestion of the α subunit of rat renal cortex (not shown).

Characterization of Antibodies Specific for the Phosphorylated Form of the Na+,K+-ATPase α Subunit—Na+,K+-ATPase α subunit from the shark rectal gland or from the rat renal cortex was phosphorylated in vitro with protein kinase A in the presence of [γ-32P]ATP and transferred electrophoretically to nitrocellulose membranes. Antibodies prepared against the phosphorylated synthetic peptide (296–448) detected the Na+,K+-ATPase α subunit in its phosphorylated state, without exhibiting any cross-reactivity with the dephospho form (Fig. 3). The same antibodies did not detect the Na+,K+-ATPase α subunit from shark rectal gland or from rat renal cortex when the enzyme had been phosphorylated in vitro using protein
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A B

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FIG. 2. Two-dimensional phosphopeptide mapping of Na⁺,K⁺-ATPase α subunit and α subunit peptide 936-948 after phosphorylation by protein kinase A. Purified Na⁺,K⁺-ATPase from shark rectal gland and synthetic peptide were phosphorylated with protein kinase A and digested with thermolysin (see "Experimental Procedures"). The autoradiograms show two-dimensional phosphopeptide maps of thermolytic digest of phosphorylated Na⁺,K⁺-ATPase (panel A), phosphorylated α subunit peptide 936-948 (panel B), and a combination of the two (panel C). Peptide 2 in panels B and C co-migrated with undigested synthetic peptide (not shown). Virtually identical results were obtained when purified Na⁺,K⁺-ATPase from rat renal cortex was used instead of Na⁺,K⁺-ATPase from shark rectal gland.

Comparison of Wild-type and Mutant Na⁺,K⁺-ATPase Transfected into COS Cells—To assess the possible physiological role in intact cells of the phosphorylation of Ser⁹⁴³ of Na⁺,K⁺-ATPase by protein kinase A, COS cells were transfected with either of 2 forms of the α subunit of rat Na⁺,K⁺-ATPase, namely the wild-type form or a mutated form in which an Ala was substituted for Ser⁹⁴³. Na⁺,K⁺-ATPase activity was assayed at various concentrations of Na⁺, using membranes isolated from these transfected cells. Under control conditions, the concentration of Na⁺ required for half-maximal activation of the enzyme (K₀.₅) was similar for the two types of transfected cells (Table I). Due to a difference in the efficacy of transfection, the Vₘₐₓ value of Na⁺,K⁺-ATPase activity in wild-type cells was lower than in mutant cells. Incubation of wild-type cells with 10 μM forskolin plus 0.5 mM IBMX caused a significant decrease in Na⁺,K⁺-ATPase activity, manifested both as a decrease in Vₘₐₓ and in the affinity of the enzyme for Na⁺ (Table I). In contrast, in membranes from mutant cells, no significant differences in either Vₘₐₓ or Na⁺ affinity were observed following forskolin plus IBMX treatment (Table I).

Incubation of wild-type cells with the selective protein kinase A inhibitor, H89 (20), abolished the inhibitory effect of forskolin plus IBMX on Na⁺,K⁺-ATPase activity observed in membranes from wild-type cells (data not shown), supporting the idea that forskolin inhibits Na⁺,K⁺-ATPase activity in wild-type cells by virtue of stimulating the phosphorylation of Na⁺,K⁺-ATPase on Ser⁹⁴³.

DISCUSSION

In kidney and brain, regulation of the state of phosphorylation of Na⁺,K⁺-ATPase is thought to play an important role in the modulation of the activity of this enzyme (6-8). Protein kinase A inhibits the activity of purified Na⁺,K⁺-ATPase by directly phosphorylating its α subunit (10). Furthermore, protein kinase A has been proposed to modulate the activity of Na⁺,K⁺-ATPase in vivo, as measured in several systems (5, 21, 27-33).

In the present study, we show that Ser⁹⁴³ is the site phosphorylated by protein kinase A in the rat kidney α1 isoform of Na⁺,K⁺-ATPase. Radiosequence analysis of the ³²P-phosphorylated Na⁺,K⁺-ATPase α subunit, after digestion with endo-protease Lys-C, indicated that Ser⁹⁴³ was a possible site for protein kinase A-dependent phosphorylation. A 13-amino acid peptide corresponding to amino acid residues 936-948 of the rat α1 isoform was found to be a very good substrate for seryl phosphorylation by protein kinase A, lending further support to the notion that Ser⁹⁴³ is the phosphorylation site for protein kinase A. In agreement with these data, two-dimensional phosphopeptide mapping experiments demonstrated that an identical phosphopeptide is generated by thermolysis cleavage either of Na⁺,K⁺-ATPase or of the synthetic peptide after phosphorylation by protein kinase A. Because Ser⁹⁴³ is located between two thermolytic cleavage sites that are present in the

kinase C (data not shown). These results support the conclusion that Ser⁹⁴³ was indeed the phosphorylation site for protein kinase A on the Na⁺,K⁺-ATPase.
ATPase by protein kinase A. Purified Na⁺,K⁺-ATPase from shark rectal gland or rat renal cortex was incubated with [γ-32P]ATP in the presence or absence of protein kinase A, as indicated. The samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose filters as described under “Experimental Procedures.” Panel A, autoradiography showing 32P-incorporation into the Na⁺,K⁺-ATPase subunit. Panel B, Western blot obtained from the same experiment using antibodies specific for the protein kinase A-phosphorylated α subunit of Na⁺,K⁺-ATPase (see “Results”).

Table 1

<table>
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<th>Cell type</th>
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<th>K_{0.5}, Na⁺ (mM)</th>
<th>V_{max} (nmol P/mg protein/h)</th>
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<td>8.8 ± 1.6 (5)</td>
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<td>Mutant Control</td>
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<td>5488 ± 286 (5)</td>
</tr>
<tr>
<td>Forskolin + IBMX</td>
<td></td>
<td>6.0 ± 0.5 (4)</td>
<td>5650 ± 371 (5)</td>
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* Significantly different versus wild-type control, p < 0.05.
* p < 0.01, Student's t test.

To prove an involvement of Ser⁹⁴³ in the regulation of the activity of Na⁺,K⁺-ATPase in vivo, a series of experiments were performed using COS cells, in which Ser⁹⁴³ was mutated to an Ala. Exposure of cells transfected with wild-type Na⁺,K⁺-ATPase to forskolin and IBMX resulted in a significant inhibition of Na⁺,K⁺-ATPase activity, measured both as an increase in K_{0.5} for Na⁺ and as a decrease in V_{max}. Such an inhibition is mediated by activation of protein kinase A, because preincubation of COS cells with the specific inhibitor of protein kinase A, H89, completely blocked the effect of forskolin and IBMX. When COS cells were used, in which Ser⁹⁴³ on the α subunit of Na⁺,K⁺-ATPase was substituted by Ala, the mutation prevented the effect of forskolin and IBMX, indicating that inhibition of the activity of Na⁺,K⁺-ATPase is achieved in vivo by phosphorylation of the seryl residue in position 943 on the α subunit. The inhibitory effect of forskolin in wild-type COS cells was less than the inhibitory effect observed upon stoichiometric phosphorylation of purified Na⁺,K⁺-ATPase by protein kinase A (10). This difference is most likely attributable to a less than stoichiometric phosphorylation of the enzyme in vivo.

The present results provide direct evidence in support of the idea that some first messengers regulate the activity of Na⁺,K⁺-ATPase by virtue of altering the state of phosphorylation of this enzyme. The α-adrenergic agonist oxymetazoline increases the affinity of Na⁺,K⁺-ATPase for Na⁺ (6); this effect is reversed by dopamine (7). It has been proposed that the opposing effects of dopamine and oxymetazoline are achieved through phosphorylation and dephosphorylation of Na⁺,K⁺-ATPase catalyzed, respectively, by protein kinase A and calcineurin. The alteration in Na⁺ affinity of Na⁺,K⁺-ATPase, which results from a change in its state of phosphorylation, can be predicted to have marked effects both on the intracellular concentration of Na⁺ and, given the electrogenic nature of the Na⁺,K⁺-
ATPase, on the membrane potential as well.

Protein kinase C has also been shown to inhibit the activity of purified Na+,K+-ATPase by directly phosphorylating its α subunit (10). It will be important to carry out experiments analogous to those reported here to determine whether the regulation of Na+,K+-ATPase by protein kinase C is of physiological relevance. This task will necessitate identifying the seryl and threonyl residues phosphorylated by protein kinase C, mutating those phosphorylation sites to Ala, transfecting the wild-type Na+,K+-ATPase.

REFERENCES