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Phosphorylation by protein kinase C of serine-23 of the α-1 subunit of rat Na^+,K^+-ATPase affects its conformational equilibrium

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ABSTRACT **Phosphorylation of the α-1 subunit of rat Na^+,K^+-ATPase by protein kinase C has been shown previously to decrease the activity of the enzyme *in vitro*. We have now undertaken an investigation of the mechanism by which this inhibition occurs. Analysis of the phosphorylation of recombinant glutathione *S*-transferase fusion proteins containing putative cytoplasmic domains of the protein, site-directed mutagenesis, and two-dimensional peptide mapping indicated that protein kinase C phosphorylated the α-1 subunit of the rat Na^+,K^+-ATPase within the extreme NH_2-terminal domain, on serine-23. The phosphorylation of this residue resulted in a shift in the equilibrium toward the E1 form, as measured by eosin fluorescence studies, and this was associated with a decrease in the apparent K^+ affinity of the enzyme, as measured by ATPase activity assays. The rate of transition from E2 to E1 was apparently unaffected by phosphorylation by protein kinase C. These results, together with previous studies that examined the effects of tryptic digestion of Na^+,K^+-ATPase, suggest that the NH_2-terminal domain of the α-1 subunit, including serine-23, is involved in regulating the activity of the enzyme.**

Na^+,K^+-ATPase is an integral membrane protein believed to be present in all animal cells. The enzyme transports three Na^+ out of the cell and two K^+ into the cell for each ATP molecule hydrolyzed. Thus the enzyme contributes to the creation of transmembrane potential, cell volume control, and the Na^+-dependent transport of protons, other ions, sugars, and amino acids (1). Na^+,K^+-ATPase activity has been shown to be dynamically regulated in a number of tissues by hormones and neurotransmitters through activation of second messenger-dependent protein kinases (2–3). It has also been shown that the α-1 subunit of Na^+,K^+-ATPase is a substrate for both cAMP-dependent protein kinase and protein kinase C (PKC) *in vitro* and that the α-1 subunit is also phosphorylated by PKC in intact cells (4–10).

cAMP-dependent protein kinase phosphorylates the rat α-1 subunit of Na^+,K^+-ATPase at Ser-943, and this is associated with a decreased apparent affinity for Na^+ and a decreased V_{max} (11). Phosphorylation of the enzyme by PKC is associated with inhibition of ATPase activity *in vitro* (4), in the OK cell line (6), and in the the choroid plexus (9), but the mechanism by which this inhibition occurs is unknown. To elucidate the mechanism of enzyme inhibition by PKC, we have carried out studies to identify the residue(s) phosphorylated by this kinase. In agreement with recent studies (10), we found that PKC phosphorylates the rat α-1 subunit of Na^+,K^+-ATPase at Ser-23 *in vitro*. Furthermore, we have examined the effect of phosphorylation of this NH_2-terminal residue on certain conformational transitions that occur during the Na^+,K^+-ATPase enzymatic cycle. The operational cycle of Na^+,K^+-ATPase is a sequence of transitions between two major conformations (termed E1 and E2) of the enzyme with different affinities for the translocated cations and ATP (12). The E1 conformation has a high affinity for MgATP and is characteristic of the enzyme in Na^+-containing media. In contrast, the E2 conformation is favored in K^+-containing media and has a low affinity for the nucleotide. The results obtained in the present study indicate that phosphorylation by PKC shifts the E1–E2 equilibrium toward the E1 form, resulting in a decrease in the apparent affinity for K^+. Thus, these changes may be responsible for the inhibition of Na^+,K^+-ATPase following phosphorylation of Ser-23 by PKC and support the idea (13–15) that the NH_2-terminal domain of the α-1 subunit is involved in regulating the activity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Eosin was from Molecular Probes. PKC (specific activity, 0.05 μmol of P_i/min/mg using histone III as substrate), purified from rat brain as described (16), was provided by Atsuko Horiuchi (The Rockefeller University).

Purification of Na^+,K^+-ATPase. Na^+,K^+-ATPase was prepared from rat renal cortex as described (17). To estimate the purity of Na^+,K^+-ATPase, samples were analyzed by electrophoresis using a 7.5% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue and by densitometric analysis. Total protein concentration was determined using the BCA method (Pierce) with bovine serum albumin as the standard. The Na^+,K^+-ATPase consistently constituted 55–60% of total protein. Based on the purity of Na^+,K^+-ATPase, the specific activity of the preparation used was calculated as approximately 600 μmol of ATP hydrolyzed per mg of protein per hour at 37°C in the presence of 120 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, and 4 mM ATP (pH 7.4).

Phosphorylation of Purified Na^+,K^+-ATPase α-Subunit by PKC *in Vitro*. Phosphorylation was carried out at 30°C for 7 min in a reaction volume of 50 μl containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, 100 μM [^{32}P]ATP (1–2000 cpm/pmol), and 500 ng of PKC. The reaction was stopped by the addition of SDS sample buffer (18). Samples were analyzed by electrophoresis using a 7.5% SDS-polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue, destained, and dried and analyzed by autoradiography. The band corresponding to the phosphorylated Na^+,K^+-ATPase α-subunit was cut from the gel, and radioactivity was measured by Cerenkov counting.

To calculate the stoichiometry of phosphorylation, the amount of the α subunit of Na^+,K^+-ATPase was measured by amino acid analysis. After transferring proteins to Immobilon PSQ in 10 mM 3-(cyclohexylamino)propanesulfonic acid (CAPS)/10% MeOH (pH 11), staining with Ponceau S and

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Abbreviations: PKC, protein kinase C; pNPPase, *p*-nitrophenylphosphatase; GST, glutathione *S*-transferase; NCF, NH_2-terminal cytoplasmic fragment; FCL, first cytoplasmic loop; SCL, second cytoplasmic loop.

autoradiography, radioactive α-subunit bands were cut and counted. In parallel, samples of dephosphorylated Na^+,K^+-ATPase were processed in an identical fashion, and the α-subunit bands were submitted for total amino acid analysis and NH_2-terminal sequence determination. The NH_2-terminal sequence corresponded to that of only the rat α-1 subunit (XXDKYEPAAVSE), and the amino acid analysis corresponded to that expected for the α-1 subunit.

For preparative phosphorylation of Na^+,K^+-ATPase for fluorescence studies and determination of the Na^+,K^+-ATPase and K^+-dependent *p*-nitrophenylphosphatase (pNPPase) activities, the conditions used were the same as described above except that the incubation time was 1 h, the temperature was 22°C, and the reaction volume was 1.5 ml. The reaction was initiated by the addition of cold ATP in the absence or presence of 1 mM $CaCl_2$ and terminated by cooling and the addition of EDTA to a final concentration of 2 mM. As a control, Na^+,K^+-ATPase was incubated under identical conditions, except that PKC was replaced by the same volume of BSA.

Phosphorylation of glutathione *S*-transferase (GST) fusion proteins (0.5-2 μg) was performed as described above except that 1.5 mM $CaCl_2$, 1 mM EGTA, 50 μg/ml phosphatidylserine, 5 μg/ml diolein, and 25–500 ng of PKC were added and the incubation was for 15 min at 30°C. Samples were subjected to SDS/PAGE (10–20% acrylamide/Tricine, NOVEX, San Diego).

Construction of Na^+,K^+-ATPase/GST Fusion Proteins in *Escherichia coli*. To facilitate the in-frame cloning of fragments encoding major intracellular domains of Na^+,K^+-ATPase α-subunit, these regions were amplified by PCR. The three domains analyzed were (numbers correspond to the initiation methionine as residue 1): NH_2-terminal cytoplasmic fragment (NCF), residues 8–92; first cytoplasmic loop (FCL), residues 148–287; second cytoplasmic loop (SCL), residues 344–777. The template used for PCR was rat Na^+,K^+-ATPase α-1 subunit cDNA (a kind gift of J. Lingrel, ref. 19) and the following pairs of primers: NCF (1), 5′-GTTGGTCTAGACAAGTATGAGCCCG-3′ and (2), 5′-GAAAAGCTTCCGTCAGAATTTGACCC-3′; FCL (3), 5′-GGCTGGATCCCCTATTATCAAG-3′ and (4), 5′-GAGGTGGATGAAGTGCTCGAATTCTTC-3′; SCL (5), 5′-CACGGGAATTCTGACGCTCACTGCC-3′ and (6), 5′-CCGGAAGCTTACTTGTTAGGGTTTAAGC-3′.

After digestion with the appropriate restriction enzymes, the fragments were cloned into the *Xba*I–*Hin*dIII sites (NCF), *Bam*HI-*Eco*RI sites (FCL), and *Eco*RI-*Hin*dIII sites (SCL), of pGEX-5x-1 (FCL) or pGEX-KG (a modified bacterial GST fusion protein expression vector) (20–21) (NCF and SCL). The fidelity of the final expression constructs was confirmed by DNA sequencing using United States Biochemical Sequenase II.

To introduce mutations in the NH_2-terminal cytoplasmic domain, a two-step PCR-based targeted mutagenesis procedure (22) was used employing oligonucleotides 1 and 2 and two additional PCR primers: (7), 5′-TGGTCTAGACAAGTATGAGCCCGCAGCTGTAGCAG-3′, introduced a mutation changing Ser-16 to Ala-16 and created a unique *Xba*I site; antisense primer (8), 5′-CTTGGCCTTCTTGTCCCCATG-3′, introduced a mutation changing Ser-23 to Ala-23. The first PCR reaction employed oligonucleotides 1 and 8 (to mutate Ser-23) or oligonucleotides 7 and 8 (to mutate both Ser-16 and Ser-23). PCR conditions were as described above. First-step PCR products were isolated from 4% agarose gel and used as a 5′ primer in a second PCR reaction employing oligonucleotide 2 as the antisense primer. Ser-16 was mutated to Ala-16 as a result of a single PCR reaction using oligonucleotides 7 and 2. After digestion with *Hin*dIII and *Xba*I, the final PCR products were cloned in pGEX-KG vector digested with the same enzymes.

Production and Purification of Recombinant Na^+,K^+-ATPase Domains. The protocol used to prepare GST fusion proteins was essentially as described (21). In some experiments, recombinant proteins were cleaved with bovine thrombin (5 mg/ml) for 3 h at 4°C, following which glutathione-Sepharose beads containing the attached GST moiety were removed by centrifugation. To remove thrombin, the NaCl concentration in the supernatant was adjusted to 0.5 M and 1/10 volume of benzamidine-Sepharose beads (Pharmacia) preequilibrated in 50 mM Tris·HCl (pH 8.0), 5 mM $CaCl_2$, 0.5 M NaCl was added. All recombinant proteins digested with thrombin included an NH_2-terminal 13-amino acid stretch (GSPGISGGGGGIL) derived from the expression vector.

Two-Dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis. Phosphopeptide map analysis was performed on excised gel slices essentially as described (11), except that trypsin or thermolysin were used at a final concentration of 0.15 mg/ml. Phosphoamino acid analysis was performed as described (23).

Na^+,K^+-ATPase and K^+-Dependent Phosphatase Activity (K^+-pNPPase) Assay. Na^+,K^+-ATPase activity was assayed by measuring the hydrolysis of [γ-^{32}P]ATP at 37°C in the presence of 120 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, and 4 mM ATP (24). The K^+ dependence of Na^+,K^+-ATPase activity was determined at 40 mM NaCl, and the apparent affinity for K^+ and the Hill coefficient were determined using a nonlinear least squares method. Na^+,K^+-ATPase catalyzes ouabain-sensitive K^+-activated hydrolysis of *p*-nitrophenyl phosphate in the absence of Na^+, and the product of the K^+-pNPPase reaction, *p*-nitrophenyl phosphate, was measured as described (25).

Fluorescence Studies. Some of the substances required for the phosphorylation by PKC, especially ATP and Mg^{2+}, complicated the fluorescence measurements with eosin (see below) and were therefore removed by dilution and centrifugation. The PKC phosphorylation medium (1.5 ml) was diluted to 27 ml with a buffer containing 10 mM Hepes/2 mM EDTA (pH 7.3) and subsequently centrifuged at 145,000 × *g* for 45 min at 4°C (repeated twice). This procedure removed essentially all interfering substances. The phosphorylated and the control enzyme were treated identically, except that in the control sample, PKC was absent. The washed enzyme retained 50% of the Na^+,K^+-ATPase activity. This washing procedure was only used for the fluorescence studies.

Eosin fluorescence was monitored using a SPEX fluorometer (slit width, 10 nm) with an excitation wavelength of 530 nm and an emission wavelength of 560 nm. The sample (0.5 ml) was continuously stirred, and the temperature was controlled by a circulating water bath at 22°C. Sample buffer contained 10 mM Hepes, 2 mM EDTA (pH 7.3), 0.2 μM eosin, and 0.02 mg/ml Na^+,K^+-ATPase. The effect of the ligands Na^+ and K^+ was determined by the addition of 1-μl aliquots of concentrated NaCl or KCl solutions to the cuvette. The time increment constant for the SPEX fluorometer was 0.3 s, and the integration time was 0.1 s. Data were analyzed and displayed using the programs ORIGIN (Microcal, Amherst, MA) and TECHGRAPHPAD (version 4.0, Binary Engineering Software, Waltham, MA) as well as the SPEX fluorometer software.

RESULTS

Phosphorylation of Serine-23 of α-1 Subunit of Rat Kidney Na^+,K^+-ATPase by PKC *in Vitro*. Previous studies of the phosphorylation of shark rectal gland Na^+,K^+-ATPase by PKC indicated that the α-subunit was phosphorylated on serine and threonine residues (4). In the present study, the phosphorylation of the α-1 subunit of rat kidney Na^+,K^+-ATPase by PKC was examined *in vitro*. PKC catalyzed the phosphorylation of the catalytic α-subunit, but not the regulatory β-subunit of rat Na^+,K^+-ATPase. Phosphoamino acid

analysis indicated that serine was exclusively phosphorylated (Fig. 1*A*). Two-dimensional phosphopeptide mapping following digestion with trypsin revealed the presence of two prominent phosphopeptides (peptides 1 and 2) (Fig. 1*B*).

Time course studies (data not shown) indicated that PKC phosphorylated the α-subunit of Na^+,K^+-ATPase rapidly (a maximal level was achieved in the first 3 min of incubation) to a stoichiometry of ~0.5 mol of P_i per mol of Na^+,K^+-ATPase α subunit. In the absence of Ca^{2+}, incorporation of ^{32}P into the α-subunit was barely detectable (data not shown). Phosphorylation by PKC did not require exogenous phosphatidylserine/diolein (data not shown). Previous results have suggested that the addition of either ouabain or phosphate is able to significantly enhance the phosphorylation of rat Na^+,K^+-ATPase by PKC (8). In our hands ouabain had small and variable effects, increasing the maximal stoichiometry from 0.5 to 0.65 (data not shown).

The major cytoplasmic domains of the α-1 subunit of rat Na^+,K^+-ATPase were expressed in *E. coli* as GST fusion proteins, and their ability to serve as substrates for PKC was examined. The regions examined corresponded to the NH_2-terminal cytoplasmic fragment (residues 8–92, NCF), the first cytoplasmic loop (residues 148–287, FCL), and the second cytoplasmic loop (residues 344–777, SCL). The NCF–GST fusion protein (Fig. 1*A*) and the FCL–GST fusion protein (data not shown) were both efficiently phosphorylated on serine residues using a low concentration of PKC (25 ng per reaction). In contrast, the SCL-fusion protein was not phosphorylated even at high concentrations of PKC (up to 500 ng per reaction mixture, data not shown). The phosphorylated NCF- and FCL-fusion proteins were subjected to two-dimensional phosphopeptide mapping using either trypsin or thermolysin. Analysis of the peptide maps revealed that peptides 1 and 2 from the α-subunit were also derived from the NCF fusion protein, (Fig. 1*B*), but not from the FCL fusion protein, indicating that the seryl residue(s) phosphorylated by PKC were present within amino acids 8–92.

Analysis of the primary structure of the NH_2-terminal cytoplasmic domain of rat Na^+,K^+-ATPase α-1 subunit (residues 8–92) (19) indicated the presence of several serine residues that corresponded to potential sites for phosphorylation by PKC (26). The predicted theoretical mobilities of thermolytic digestion products of the NH_2-terminal region of the rat α-1 Na^+,K^+-ATPase suggested that one very basic peptide could only be derived from amino acid residues 15–34 (VSEHGDKKSKKAKKERDMDE).

To determine whether Ser-16 or Ser-23 was phosphorylated by PKC, three different mutant forms of NCF were prepared in which Ser-16, Ser-23, or both Ser-16 and Ser-23, were mutated to alanine. Equal amounts of wild-type NCF and each of the three mutant proteins were phosphorylated by PKC (Fig. 1*C*). Recombinant proteins in which Ser-23 was mutated to Ala-23 were not significantly phosphorylated by PKC. In contrast, mutation of Ser-16 to Ala-16 had no effect on phosphorylation of the mutant protein. The NCF and NCF (S16A) fusion proteins were digested with trypsin and subjected to two-dimensional phosphopeptide mapping (Fig. 1*B*). Analysis of the peptide maps revealed peptides 1 and 2. Therefore, these results indicate that Ser-23 represents the major site for phosphorylation by PKC in rat Na^+,K^+-ATPase α-1 subunit.

Effect of Phosphorylation of Na^+,K^+-ATPase on Eosin Fluorescence. The equilibrium and the transitions between the two conformational states E1 and E2 were probed with the fluorescent dye eosin. Eosin binds with high affinity to the nucleotide binding site of the pump in the presence of Na^+, when the enzyme is in the E1 form (27). The fluorescence of eosin is enhanced severalfold upon binding. Addition of K^+, which converts the enzyme into the E2 form, leads to disso-

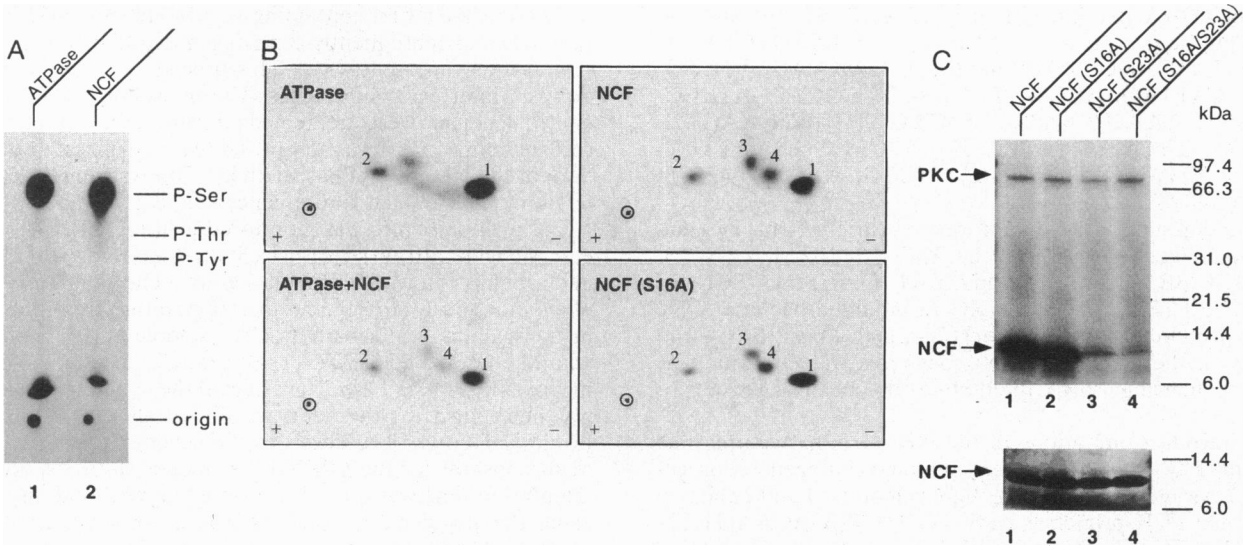

FIG. 1. Phosphorylation of Ser-23 of rat Na^+,K^+-ATPase α-1 subunit by PKC. (*A*) Autoradiogram showing phosphoamino acid analysis of rat Na^+,K^+-ATPase α-1 subunit and recombinant wild-type NCF phosphorylated by PKC. The positions of unlabeled phosphoamino acid standards are indicated. The labeled spots close to the origin represent unhydrolyzed peptide. (*B*) Two-dimensional tryptic phosphopeptide mapping of rat Na^+,K^+-ATPase α-subunit and recombinant proteins. Rat α-1 Na^+,K^+-ATPase was phosphorylated by PKC, the samples were analyzed by SDS/PAGE (not shown), and the band containing the α-1 subunit was excised from the gel and digested with trypsin. Radioactive bands containing NCF or NCF in which Ser-16 was mutated to Ala-16, from the experiment shown in Fig. 1*C*, were excised and digested with trypsin. ATPase, whole α-1 subunit; NCF, recombinant wild-type NCF; ATPase + NCF, whole α-1 subunit plus recombinant wild-type NCF; NCF (S16A), NCF in which Ser-16 was mutated to Ala-16. Electrophoresis was performed in horizontal direction (O, origin; positive electrode at left) and chromatography was performed in the vertical direction. Dried sheets vere subjected to autoradiography. Peptides 1 and 2 from holo α-1 subunit or NCF include Ser-23. Peptides 3 and 4 are derived from unidentified serine residues phosphorylated by PKC only in the recombinant protein. (*C*) Equal amounts of wild-type NCF and NCF in which Ser-16 was mutated to Ala-16 (S16A), Ser-23 was mutated to Ala-23 (S23A), or both Ser-16 and Ser-23 were mutated to Ala-16 and Ala-23 (S16A/S23A), were phosphorylated using PKC as described in *Experimental Procedures*. Phosphorylated samples were analyzed by SDS/PAGE followed by silver staining (bottom panel) and autoradiography (top panel). Arrows show the positions of NCF recombinant proteins and autophosphorylated PKC. Numbers on the right indicate the positions of molecular mass markers.

ciation of eosin from the nucleotide binding site, and thus to a decrease in fluorescence.

The rate of transition from E1 to E2 is so fast that it cannot be followed continuously during the addition of a single saturating dose of KCl. The transition to the E2 form was therefore studied by titration with subsaturating doses of KCl. A typical experiment is shown in Fig. 2. Following each addition of KCl, there was a decrease in fluorescence to a new equilibrium level. For each experiment, the half-maximal decrease in eosin fluorescence, $K_{0.5}$, for K^+ was calculated following a hyperbolic fit of the data. The transition from the E1 to the E2 form was compared for the phosphorylated and nonphosphorylated, but otherwise identically treated, enzyme in six paired experiments. Data points from all experiments are shown in Fig. 3. The $K_{0.5}$ for K^+ was 2.15 ± 0.10 (n = 6) for Na^+,K^+-ATPase phosphorylated by PKC and for the washed, control enzyme 1.20 ± 0.10. This difference was highly significant ($P < 0.001$, paired experiments).

To examine the effect of the washing procedure on the equilibrium between E1 and E2, the enzyme was exposed to the phosphorylation buffer (but with no PKC present) and subsequently washed. The $K_{0.5}$ (K^+) values were 1.37 ± 0.08 for native enzyme (n = 5) and 1.20 ± 0.10 (n = 6) for washed enzyme, $P > 0.05$. As an additional control experiment for the effect of PKC, Na^+,K^+-ATPase was treated with PKC as described above, but in the absence of Ca^{2+}. The $K_{0.5}$ (K^+) for this preparation and for washed Na^+,K^+-ATPase were not significantly different (data not shown), indicating that it was the phosphorylation *per se*, which affected the conformational equilibrium.

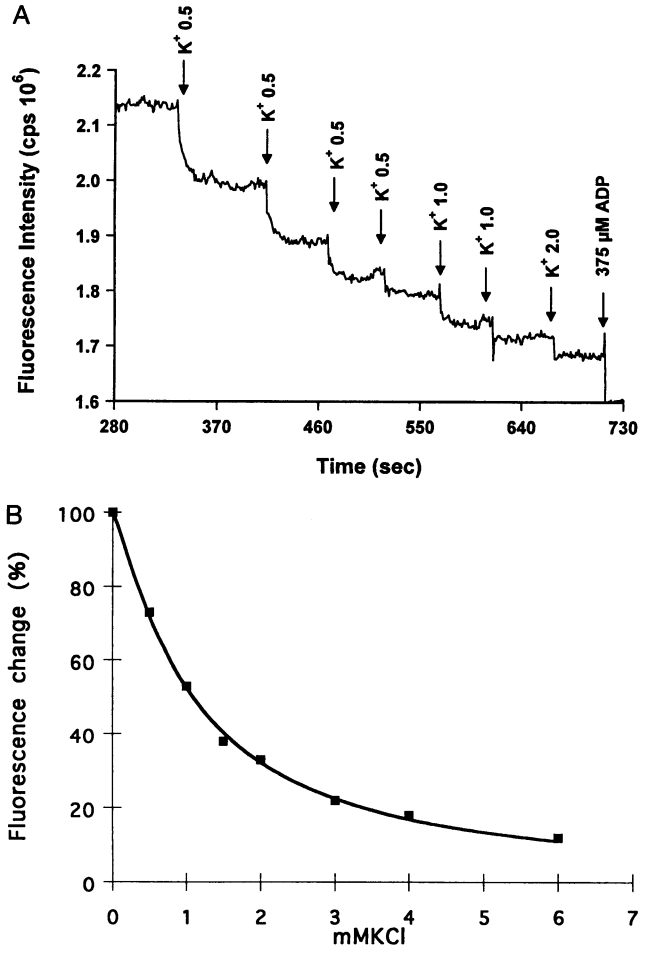

FIG. 2. Titration of the Na^+,K^+-ATPase E1-Na^+ eosin fluorescence with KCl. (*A*) Purified rat kidney Na^+,K^+-ATPase (0.02 mg/ml) in a buffer containing 10 mM Hepes, 2 mM EDTA (pH 7.3), 30 mM NaCl, and 0.2 μM eosin, was titrated with different concentrations of KCl (in mM). The eosin fluorescence was measured as described (28). After the last addition of KCl, a zero level of the eosin fluorescence was achieved in the presence of 0.375 mM ADP, which displaces all eosin from the binding sites. (*B*) The change in eosin fluorescence as a function of [KCl] is given as percent of the total fluorescence response caused by eosin binding. The line is drawn according to the Hill equation with n = 1.0: $F = 100 - 100\cdot[K^+]^n/([K^+]^n + K_{0.5}^n)$.

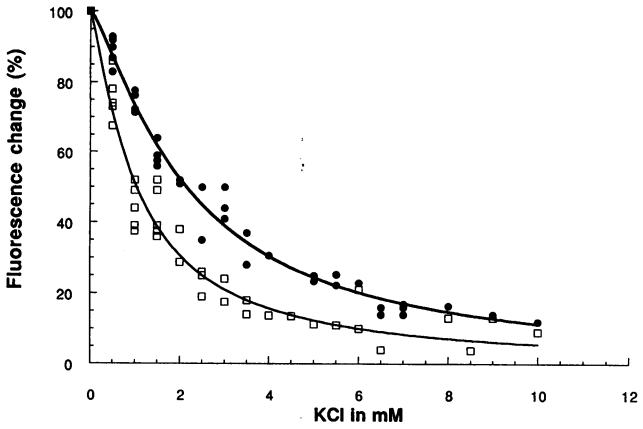

FIG. 3. Effect of PKC phosphorylation of Na^+,K^+-ATPase on the apparent affinity for K^+ measured by eosin fluorescence. Eosin fluorescence is shown as a function of KCl concentration for the washed control enzyme (open squares) and the enzyme that had been phosphorylated by PKC and then washed (filled circles). Here the data for PKC-phosphorylated enzyme have been analyzed as in Fig. 2. In six paired experiments $K_{0.5}$ for K^+ are 1.20 ± 0.10 with a Hill coefficient of 1.2 (control enzyme) and 2.15 ± 0.05 with a Hill coefficient 1.3 (phosphorylated enzyme).

The rate constant for the conversion from E2–K^+ to E1–Na^+ is so slow that the reaction can be followed in a conventional fluorometer by the addition of a single dose of 30 mM NaCl, which turns almost all enzyme molecules into the E1 form. A typical experiment is shown in Fig. 4. There was no apparent effect of the phosphorylation by PKC on the E2 to E1 rate constant, which in three paired experiments averaged 0.177 ± 0.019 s^{-1} for the phosphorylated enzyme and 0.163 ± 0.012 s^{-1} for the enzyme exposed to PKC buffer with subsequent washing. Nor was there any effect of the washing procedure on the E2–K^+ to E1–Na^+ conversion (data not shown).

Effect of PKC Phosphorylation on Na^+,K^+-ATPase and K^+-Dependent Phosphatase Activities. ATP hydrolysis was measured in the presence of 40 mM NaCl and various concentrations of K^+. Phosphorylation by PKC decreased Na^+,K^+-ATPase activity at all concentrations of KCl studied. The V_{max} decreased from 494 ± 21 to 337 ± 20 μmol P_i per mg of protein per hr and the $K_{0.5}$ (K^+) increased from 0.23 ± 0.02 to 0.35 ± 0.05 in 12 paired experiments. The K^+-dependent phosphatase activity was measured in the presence of 150 mM KCl and in the absence of Na^+ with *p*-nitrophenyl phosphate as a substrate. K^+-pNPPase activity was significantly lower for phosphorylated enzyme than for enzyme that had been exposed to phosphorylation buffer. The mean values in five experiments were 154 ± 4 and 105 ± 6 μmol of P_i per mg of protein per hr, respectively.

DISCUSSION

The present studies indicate that phosphorylation of the rat α-1 subunit of Na^+,K^+-ATPase occurs on the NH_2-terminal cytoplasmic region (Ser-23) and is associated with a change in the conformational equilibrium of the enzyme. Recent studies in intact cells have shown that PKC phosphorylates the rat α-1 subunit of Na^+,K^+-ATPase and that this is associated with

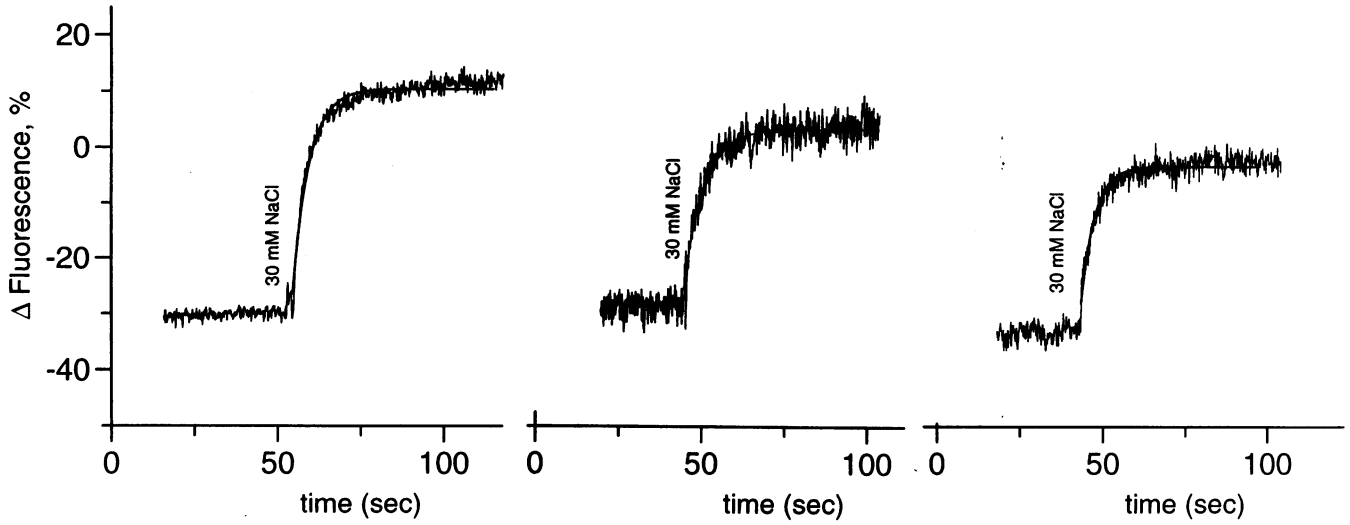

FIG. 4. Time course of eosin fluorescence changes induced by Na^+. Thirty mM NaCl was added to rat kidney Na^+,K^+-ATPase (0.03 mg/ml in a buffer containing 10 mM Hepes, 2 mM EDTA (pH 7.3), 1 mM KCl, and 0.2 μM eosin) at time zero, and the fluorescence of eosin was monitored as a function of time. The left panel shows a typical experiment with native enzyme; the center panel shows an experiment with washed enzyme; and the right panel shows an experiment with enzyme phosphorylated by PKC. The solid lines indicate single-exponential curves with rate constants of 0.20 s^{-1} (*Left*), 0.17 s^{-1} (*Center*), and 0.19 s^{-1} (*Right*).

inhibition of enzyme activity (9). Furthermore, our present results, together with comparison of two-dimensional peptide maps (9), indicate that the site of the α subunit phosphorylated in intact cells by PKC is Ser-23. It was recently reported that the major site phosphorylated in toad (*Bufo marinus*) Na^+,K^+-ATPase α-1 subunit is Ser-16 (7). As shown in the present report, Ser-23, but not Ser-16, is predominantly phosphorylated by PKC in the rat form of the α-1 subunit. During the course of these studies, Ser-23 was also identified as a major site for PKC in the rat α-1 subunit (10). Ser-16 is conserved in α-1 subunits from both species. However, Ser-23 is absent from the *B. marinus* Na^+,K^+-ATPase. The preference for phosphorylation of Ser-23 in the rat α-1 isoform is in accordance with the fact that the surrounding amino acid sequence constitutes an excellent consensus phosphorylation site for PKC (26). In contrast, the amino acid sequence including Ser-16 does not correspond well to a PKC consensus site.

Analysis of the NH_2-terminal region in α-subunit isoforms from various species indicates considerable variability in the presence of residues equivalent to either Ser-16 or Ser-23. For example, the rat α-2 isoform contains no seryl residue within any consensus sequence for PKC. In contrast, the rat α-3 isoform contains three serine residues, which, though not present within amino acid sequences identical to those in rat α-1, are potential phosphorylation sites for PKC. Previous results have shown that the α-subunit from several species including shark rectal gland, duck salt gland, and *B. marinus* is phosphorylated by PKC on both serine and threonine residues (4–5, 7). In the case of *B. marinus*, Thr-15 has been identified as a second site for PKC (7). Together, these results raise the strong possibility that the α-1 subunits of Na^+,K^+-ATPase from different species or different isoforms from a single species may be differentially regulated by phosphorylation by PKC. This would provide a possible explanation for previous results of the phosphorylation and regulation of Na^+,K^+-ATPase by PKC. For example, species differences in the modulation of the voltage-dependence of Na^+,K^+-ATPase by diacylglycerol analogs have been shown (28). Phosphorylation of Na^+,K^+-ATPase has been correlated with inhibition of activity in response to PKC activation in opossum kidney cells (6) and rat choroid plexus (9). In contrast, no phosphorylation and no inhibition of Na^+,K^+-ATPase activity was found in pig kidney cells in response to PKC activation. Notably, pig α-1 subunit does not contain Ser-23.

In vitro, our previous results have shown that phosphorylation of shark rectal gland Na^+,K^+-ATPase (4) and rat α1 Na^+,K^+-ATPase (present study) by PKC is associated with inhibition of enzyme activity. In contrast, Feschenko and Sweadner (8) did not see any effect of phosphorylation on the V_{max} activity of rat Na^+,K^+-ATPase, despite a stoichiometry of 1 mol/mol. Differences in assay conditions might provide an explanation for this difference. The E1 to E2 transition and the K^+ affinity were not examined in their study.

In our study, we obtained a maximal stoichiometry of 0.5 mol/mol. The reasons for this incomplete level of phosphorylation is not known at this time. From the specific Na^+,K^+-ATPase activity of 600 μmol/mg of protein per hour observed for our preparation and the generally accepted turnover number for the Na^+,K^+-ATPase (17) of about 9000 per min, it can be inferred that only half of the enzyme molecules in our preparation were active. This raises the question of whether or not active and inactive enzyme are both phosphorylated by PKC. We believe that at least the active form of the enzyme must be phosphorylated since we observed an effect of phosphorylation on K^+ activation of activity. If only active enzyme was phosphorylated, the true stoichiometry would obviously be higher. However, if we assume that there is a random, but incomplete, phosphorylation of both active and inactive molecules, the true effects on apparent K^+ affinity and ATP hydrolysis would be larger than that calculated. At the present time we cannot distinguish between these two possibilities.

A large number of studies have provided evidence that Na^+,K^+-ATPase undergoes a series of conformational changes during its normal cycling (29). For example, tryptic digestion and studies using fluorescent probes have described a number of differences between the two major conformational forms, E1 and E2 (30). In the present study, a decrease in the apparent affinity for K^+ was observed upon phosphorylation of Na^+,K^+-ATPase by PKC. This appears to be related to an increase in the Na^+/K^+-affinity ratio for the E1 form as suggested from the equilibrium fluorescence experiments (Fig. 3) and the lack of change in the rate of transition from E2 to E1 (Fig. 4). The decrease in apparent affinity for K^+ for the E1 form upon phosphorylation by PKC can be interpreted as an increase in the concentration of the E1–Na form at a given concentration of Na^+ and K^+ (here assuming that all eosin-binding enzyme molecules are affected by PKC).

It is of interest that the NH_2-terminal region of the α-subunit was also implicated in regulation of Na^+,K^+-ATPase activity in experiments in which this region of the molecule was removed by selective tryptic cleavage (13, 30). Specifically, truncation of the first 30 NH_2-terminal amino acids of the α-1 subunit caused a decline in Na^+,K^+-ATPase and K^+-phosphatase activity and a shift in the E2/E1-equilibrium toward the E1 form. Interestingly, the decrease in the relative affinity for K^+ as a result of phosphorylation by PKC is in good agreement with results of intrinsic fluorescence studies of trypsinized (T2-truncated) Na^+,K^+-ATPase (31). Studies on chimeric Na^+,K^+- and Ca^{2+}-ATPase molecules also point to a role of the NH_2-terminal 70 amino acids in conferring Na^+ sensitivity to the pump (32). Isoforms of the α-subunit also display different sensitivities to K^+ as well as to ouabain (33). Together the results of these studies suggest that the NH_2-terminal region acts to increase Na^+,K^+-ATPase activity and that removal of the region through truncation, or a structural alteration due to phosphorylation, eliminates the ability of this region to activate the enzyme. In any event, it is clear that the NH_2 terminus plays an important role in the regulation of enzyme activity.

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