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Is Phosphorylation of the α1 Subunit at Ser-16 Involved in the Control of Na,K-ATPase Activity by Phorbol Ester–activated Protein Kinase C?

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The α1 subunit of Na,K-ATPase is phosphorylated at Ser-16 by phorbol ester-sensitive protein kinase(s) C (PKC). The role of Ser-16 phosphorylation was analyzed in COS-7 cells stably expressing wild-type or mutant (T15A/S16A and S16D-E) ouabain-resistant *Bufo* α1 subunits. In cells incubated at 37°C, phorbol 12,13-dibutyrate (PDBu) inhibited the transport activity and decreased the cell surface expression of wild-type and mutant Na,K-pumps equally (~20–30%). This effect of PDBu was mimicked by arachidonic acid and was dependent on PKC, phospholipase A_2, and cytochrome P450-dependent monooxygenase. In contrast, incubation of cells at 18°C suppressed the down-regulation of Na,K-pumps and revealed a phosphorylation-dependent stimulation of the transport activity of Na,K-ATPase. Na,K-ATPase from cells expressing α1-mutants mimicking Ser-16 phosphorylation (S16D or S16E) exhibited an increase in the apparent Na affinity. This finding was confirmed by the PDBu-induced increase in Na sensitivity of the activity of Na,K-ATPase measured in permeabilized nontransfected COS-7 cells. These results illustrate the complexity of the regulation of Na,K-ATPase α1 isozymes by phorbol ester-sensitive PKCs and reveal 1) a phosphorylation-independent decrease in cell surface expression and 2) a phosphorylation-dependent stimulation of the transport activity attributable to an increase in the apparent Na affinity.

INTRODUCTION

In animal cells, the Na,K-ATPase uses the energy of ATP hydrolysis for the countertransport of three Na and two K ions and thereby maintains the electrochemical gradients of these ions across the plasma membrane. Because this process is electrogenic, it also participates in the generation of the resting membrane potential. In addition to these general functions, Na,K-ATPase promotes the membrane repolarization in excitable cells, and it provides the driving force for vectorial Na transport in epithelial cells. The enzyme is composed of at least two subunits: the large α subunit with 10 transmembrane segments carries the catalytic and ion transport properties, and the smaller, single-membrane–spanning β subunit is involved in the maturation of Na,K-ATPase and in the modulation of its transport activity.

Because Na,K-ATPase is of crucial importance in many physiological and pathological processes, the elucidation of the mechanisms that regulate its activity is an important issue. It has recently been demonstrated that Na,K-ATPase activity can be controlled by hormones and second messengers independently of Na availability or changes in the rate of subunit synthesis (McGill and Guidotti, 1991; Féraille *et al.*, 1994, 1995; Chibalin *et al.*, 1997; Carranza *et al.*, 1998). This rapid modulation of Na,K-ATPase activity by hormones may be linked to a direct phosphorylation of the Na,K-ATPase α1 subunit by serine/threonine kinases. For instance, in purified enzyme preparations (Bertorello *et al.*, 1991; Feschenko and Sweadner, 1994), in homogenates of *Xenopus* oocytes (Chibalin *et al.*, 1992), and in intact cells (Middleton *et al.*, 1993; Béguin *et al.*, 1994; Carranza *et al.*, 1996; Feschenko and Sweadner, 1997), protein kinase C (PKC) can phosphorylate the α1 isoforms of Na,K-ATPase and to a much lesser extent the α2 and α3 isoforms (Béguin

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Abbreviations used: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; PDBu, phorbol 12,13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2.

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et al., 1996b). Two PKC phosphorylation sites have been located in the cytoplasmic NH_2 terminus of α1 subunits. The first PKC phosphorylation site identified is present in all α1 subunits and was mapped to Ser-16 in an unusual (Ser-Glu-His) PKC motif (Béguin *et al.*, 1994, 1996b). A second rat-specific α1 subunit PKC phosphorylation site was mapped to Ser-23 (Feschenko and Sweadner, 1995; Béguin *et al.*, 1996b). It should be mentioned that Ser-16 and Ser-23 numbered from the initial methionine residue are also named Ser-11 and Ser-18 by other authors according to the removal of the NH_2-terminal 5 amino acids during the processing of the α1 chains.

The functional role of PKC phosphorylation is not yet resolved. Indeed, stimulatory, inhibitory, or no effects have been attributed to PKC phosphorylation (Bertorello *et al.*, 1991; Middleton *et al.*, 1993; Fisone *et al.*, 1995; Carranza *et al.*, 1996; Feschenko and Sweadner, 1997; Pedemonte *et al.*, 1997). In view of the multiple mechanisms that affect Na,K pump activity and the possible interplay between different signaling pathways, these conflicting results suggest that tissue-specific factors and uncontrolled experimental conditions may mask the basic function of PKC phosphorylation. In addition, the possibility of specific effects mediated by phosphorylation of either Ser-16 or Ser-23 or both should be considered (Vasilets, 1997). Previous studies focusing on the functional effect of Ser-23 phosphorylation, i.e. the additional rat PKC site, have documented a phosphorylation-dependent inhibition of Na,K-ATPase activity (Belusa *et al.*, 1997; Chibalin *et al.*, 1999). In contrast, the functional role of Ser-16 phosphorylation, i.e., the ubiquitous PKC site, has not yet been defined (Beron *et al.*, 1997).

In the present study, we stably expressed the wild-type *Bufo marinus* α1 subunit or its T15A/S16A mutant in COS-7 cells. Furthermore, we studied α1-mutants in which the Ser-16 phosphorylated by PKC was replaced by negatively charged amino acids (Asp or Glu), which in other settings was shown to mimic constitutive phosphorylation (Hoffman *et al.*, 1994; Pages *et al.*, 1994). In each cell line, we analyzed the effects of the activation of phorbol ester-sensitive PKCs on the activity of Na,K-ATPase and the cell surface expression of Na,K-pumps under various experimental conditions. The α1 subunit of the *Bufo* Na,K-ATPase was chosen for transfection because 1) it forms ouabain-resistant Na,K-pumps that can be distinguished from the ouabain-sensitive, endogenous Na,K-pumps of COS cells; and 2) it is efficiently phosphorylated by PKC in intact cells (Béguin *et al.*, 1994). Finally and most importantly, besides a residual 10% phosphorylation on Thr-15, the *Bufo* α1 subunit is mainly phosphorylated by PKC on Ser-16 (Béguin *et al.*, 1996b), which is representative of all known mammalian α1 subunits.

Our results indeed reveal a complex pattern of regulatory mechanisms that affect Na,K-ATPase activity after stimulation of phorbol ester-sensitive PKCs, which is dependent on experimental conditions and thus may partially account for the contradictory results reported in the literature. However, comparison, under defined experimental conditions, of the functional properties of wild-type α1 subunits, on the one hand, and Ser-16 mutants, on the other hand, clearly demonstrates that PKC phosphorylation of Ser-16 has a basic stimulatory effect on Na,K-ATPase activity, which relies on an increase in the apparent Na affinity.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with: 10% fetal calf serum, L-glutamine (10^{-6} M), penicillin (10 IU/ml), and streptomycin (100 μg/ml). COS-7 cells were transfected with the expression vector pRK5 (a gift of J.R. Didsbury, Duke University Medical Center, Durham, NC) containing the cDNA for the wild-type *B. marinus* α1 subunit or the α1 subunit mutated at Thr-15 and/or Ser-16 (T15A/S16A or S16D-E) or at Ser-943 (S943A), as previously described (Béguin *et al.*, 1994). COS-7 cells stably expressing the wild-type or mutated α1 subunit of *B. marinus* were selected on the basis of the ouabain resistance of the *Bufo* enzyme (IC_{50}, ~5×10^{-5} M) compared with the endogenous Na,K-ATPase of COS-7 cells (IC_{50}, ~3×10^{-7} M). Two days after transfection, the medium was supplemented with 2.5×10^{-6} M ouabain and changed every 2 d, and 3 wk later, the surviving cell clones were isolated and tested for 1) the presence (wild type) or absence (T15A/S16A or S16D-E) of PKC-dependent phosphorylation of the exogenous α1 subunits (Béguin *et al.*, 1994) and 2) the presence of an ouabain-resistant ^{86}Rb uptake accounting for at least 40% of the total (endogenous and exogenous) Na,K-ATPase-mediated ^{86}Rb uptake (see below). In these stably transfected cells, the presence of functional ouabain-resistant Na,K-ATPase units in the plasma membrane is dependent on the association of the exogenous α1 subunits with the endogenous β subunits.

After stable transfection, COS-7 cells were subsequently grown in medium supplemented with 2.5×10^{-6} M ouabain to maintain the selection pressure. For experiments, cells were grown to confluence and were used between passages 10 and 30.

Na,K-ATPase-mediated ^{86}Rb Uptake

The transport activity of Na,K-ATPase was measured as the ouabain-sensitive ^{86}Rb uptake under conditions of initial rate. For this purpose, COS-7 cells were seeded on 12 multiwell plates (22-mm-diam wells) and grown to confluence. After removal of the culture medium, cells were washed twice with 1 ml of HEPES-buffered (20 mM, pH 7.4) bicarbonate- and serum-free DMEM. Cells were then preincubated at 37 or at 18°C for 15–30 min after addition of 1 ml of the same medium containing or not activators of protein kinases or/and inhibitors. ^{86}Rb uptake was determined in triplicate samples after addition of 10 μl of DMEM containing tracer amounts of ^{86}RbCl (Amersham, Little Chalfont, United Kingdom; 100 nCi/sample). The K concentration was 5.36 mM during incubation and uptake periods. Incubation was stopped after 1 min (37°C) or 15 min (18°C) by cooling on ice and rapid aspiration of the incubation medium. After three washes with 1 ml of ice-cold washing solution containing 150 mM choline-chloride, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mM $BaCl_2$, and 5 mM HEPES, pH 7.4, cells were lysed in 0.5 ml of 1% (wt/vol) Na deoxycholate, and 0.4 ml of the lysate were transferred into a counting vial and radioactivity was measured by liquid scintillation. The remaining 0.1 ml of the lysate was used to determine the protein content by the bisinchoninic acid assay (Pierce, Rockford, IL).

The total ouabain-sensitive ^{86}Rb uptake, i.e., the sum of endogenous and exogenous Na,K-ATPase-mediated Rb (K) transport, was calculated as the difference between the mean values measured in triplicate samples incubated with or without 2.5×10^{-3} M ouabain. The Rb (K) transport mediated by the exogenous Na,K pumps containing the *Bufo* α subunit was calculated as the difference between the mean values measured in triplicate samples incubated with 2.5×10^{-6} or 2.5×10^{-3} M ouabain. When present, ouabain was introduced at the beginning of the preincubation step. ^{86}Rb uptake was calculated as picomoles of Rb (K) $\times$ minute^{-1} $\times$ microgram of protein^{-1}. Preliminary experiments have shown that Rb (K) uptake was linear for at least 3 or 20 min at 37 or 18°C, respectively (our unpublished results).

Hydrolytic Activity of Na,K-ATPase

The hydrolytic activity of Na,K-ATPase was estimated by measuring the release of inorganic phosphate from [γ-^{32}P]ATP (Fisone *et al.*, 1994). Na,K-ATPase activity was measured either in situ on permeabilized cells or in crude membrane preparations.

Measurement of Na,K-ATPase activity in permeabilized cells was performed essentially as described previously (Chibalin *et al.*, 1999). COS-7 cells grown to confluence in 25-cm^2 flasks were detached by trypsinization. Trypsin was then neutralized by addition of 20 ml of DMEM supplemented with 10% (vol/vol) fetal calf serum. Cells from two flasks were pooled and resuspended in 2 ml of incubation solution containing 120 mM choline-Cl, 5 mM KCl, 4 mM $KHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM KH_2PO_4, 0.15 mM K_2HPO_4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, 20 mM HEPES, and 0.1% BSA, pH 7.45 (with KOH). Cells were then separated in two 1-ml aliquots and were preincubated at 37 or 18°C in the absence or presence of phorbol 12,13-dibutyrate (PDBu). Preincubation was stopped by cooling on ice, and cells were permeabilized by a freeze–thaw step at −20°C. Cell aliquots containing 5–15 μg of protein were transferred with 20 μl of incubation solution into 1.5-ml microtubes. After addition of 80 μl of ATPase assay solution (see below), permeabilized cells were incubated for 15 min at 37°C. The reaction was stopped by cooling on ice and addition of 1 ml of 10% (wt/vol) activated charcoal. After mixing and centrifugation, the radioactivity was measured by liquid scintillation on 250-μl aliquots of supernatants, which contain the inorganic phosphate formed from ATP. In each experiment, ATPase activities were determined on four replicates for each condition. Preliminary experiments have shown that preincubation of cells in the absence of Na does not alter the effects of PDBu on Na,K-ATPase activity (our unpublished results). It should be mentioned that the sensitivity of the method did not allow us to measure Na,K-ATPase activity in the presence of Na concentrations <5 mM.

Na,K-ATPase activity was also measured on isolated membranes prepared according to the method of Vilsen (Vasilets *et al.*, 1991) from stably transfected COS-7 cells and made leaky by sonication followed by a freeze–thawing step or by treatment with SDS, which gave similar results. All assays were carried out in the presence of 2.5 μM ouabain to inhibit the endogenous COS-7 cell Na,K-ATPase. In each experiment and for each Na concentration, ATPase activity was determined as described above on four replicates containing 5–10 μg of protein from crude membrane preparations.

The ATPase assay solutions contained various amounts of NaCl (0–140 mM), 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM Tris-HCl, 1 mM Tris-ATP, and tracer amounts (5 nCi/μl) of [γ-^{32}P]ATP (DuPont, Boston, MA; 10 Ci/mmol) for measurements of total ATPase activity. For basal Mg-ATPase activity measurements, NaCl and KCl were omitted, and 1 mM ouabain was added. The osmolarity of ATP assay solutions was adjusted by addition of choline-chloride, and the pH of each solution was 7.4. Na,K-ATPase activity was taken after subtracting the mean Mg-ATPase activity from the mean total ATPase activity and was calculated as picomoles of ATP × microgram of protein^{-1} × hour^{-1} ± SE.

Cell Surface Biotinylation, Streptavidin Precipitation, and Immunoblot

Changes in cell surface expression of Na,K-ATPase were analyzed by immunoblot after streptavidin precipitation of biotinylated cell surface proteins as described by Gottardi *et al.* (1995) with slight modifications. For this purpose, COS-7 cells were seeded on 12 multiwell plates (22-mm-diam wells) and grown to confluence. After removal of the culture medium, cells were washed twice with 1 ml of HEPES-buffered (20 mM, pH 7.4) bicarbonate- and serum-free DMEM. Cells were then preincubated at 37 or 18°C for 15–30 min after addition of 1 ml of the same medium containing or not activators of protein kinases or/and inhibitors. Incubation was stopped by cooling on ice and rapid aspiration of the incubation medium. Cells were then washed once with PBS-CM (PBS, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$) and incubated at 4°C for 1 h with biotinylation buffer (10 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, and 150 mM NaCl) containing 1.5 mg/ml biotin (EZ-Link sulfo-NHS-biotin; Pierce). After aspiration of the biotinylation buffer, cells were incubated for 20 min at 4°C in PBS-CM supplemented with 100 mM glycine to quench the unreacted sulfo-NHS-biotin, washed once with PBS-CM, and lysed in 200 μl of 1% (wt/vol) Na-deoxycholate. Equal amounts of proteins (30–50 μg) were added to 100 μl of streptavidin-agarose beads (Immunopure immobilized streptavidin; Pierce) diluted in anti-protease–containing buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10 mg/ml aprotinin, and 50 mg/ml leupeptin) supplemented with 0.5% digitonin and were incubated overnight at 4°C. The beads were then washed once with rinsing solution A (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.5% digitonin), twice with rinsing solution B (500 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.05% digitonin), three times with rinsing solution C (500 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% BSA, and 0.25% digitonin), and once with 10 mM Tris-HCl, pH 7.4. Samples were then resuspended in 100 μl of sample buffer, heated at 65°C for 15 min, and subjected to 7% SDS-PAGE. After electrotransfer, α1 subunits were revealed using a specific anti-*Bufo* α1 subunit antibody (a kind gift from Dr. François Verrey, University of Zürich, Zürich, Switzerland). This *Bufo* anti-α1 antibody did not recognize the endogenous COS-7 cell α subunit or rat or rabbit α subunits. The immunoreactivity was detected by the enhanced chemiluminescence method, according to the manufacturer's instructions (Amersham).

Preliminary experiments have shown that membranes of COS-7 cells are impermeable to sulfo-NHS-biotin, because Hsp27, an abundant cytosolic protein, was only detected by Western blotting (monoclonal antibody from StressGen, Victoria, British Columbia, Canada) in total cellular extracts but not in biotinylated and streptavidin-precipitated samples (our unpublished results).

Statistics and Calculations

For comparisons between two means expressed as absolute values, statistical analysis was done by Student's *t* test for unpaired data (^{86}Rb uptakes) or for paired data (hydrolytic activity of Na,K-ATPase) when appropriate. The Mann–Whitney *U* test was used for comparisons between two groups for data expressed as fractional changes. Comparison between more than two groups was done by analysis of variance for results expressed as absolute values or by the Kruskal–Wallis test for results expressed as fractional changes, respectively.

Values of the ouabain inhibition of ^{86}Rb uptake were fitted to the following two equations describing either a homogenous population or two independent subpopulations of Na,K-ATPase (Féraille *et al.*, 1993):

$$v = V/(1 + [O]/K_i) \quad (1)$$

or

$$v = V_1/(1 + [O]/K_{i_1}) + V_2/(1 + [O]/K_{i_2}) \quad (2)$$

where v is the ^{86}Rb uptake measured at a given concentration of ouabain ([O]); V is the ^{86}Rb uptake measured in the absence of ouabain; and K_i is the apparent inhibition constant for ouabain of the Na,K-ATPase (IC_{50}). Kinetic parameters (V and IC_{50}) were determined from Eqs. 1 and 2 by nonlinear regression analysis using Prism 2.0 software (Graphpad Software, San Diego, CA).

As previously described (Féraille *et al.*, 1995), the Na dependence of the Na,K-ATPase was analyzed using the Hill equation:

$$v = V_{max} \cdot [Na]^n([Na]^n + K_{0.5}{}^n) \quad (3)$$

where v is the Na,K-ATPase activity measured at a given concentration of Na ([Na]); V_{max} is the maximal Na,K-ATPase activity; $K_{0.5Na}$ is the apparent dissociation constant for Na; and n is the Hill coefficient.

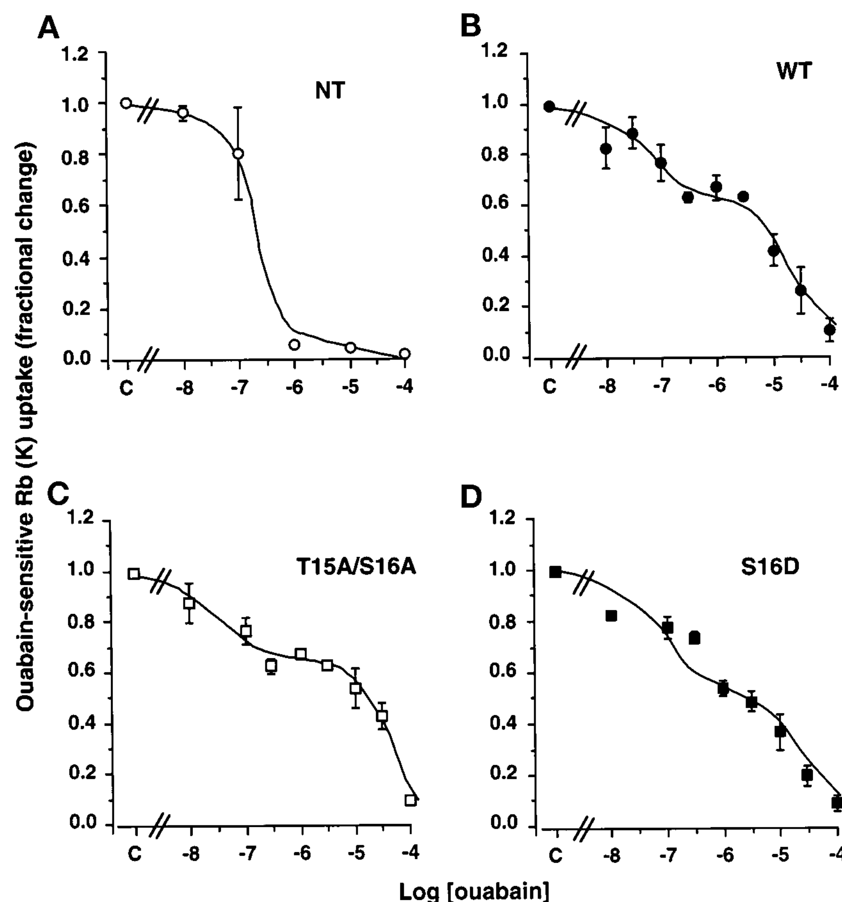

Figure 1. Stable transfection of COS-7 cells with wild-type or mutant *B. marinus* α1 subunits results in functional expression of ouabain-resistant Na,K-ATPase. ^{86}Rb uptake was measured under initial rates in COS-7 cells preincubated for 15 min at 37°C in the absence or in the presence of increasing concentrations of ouabain. The Na,K-ATPase-mediated ^{86}Rb uptake was obtained by subtraction of ouabain-insensitive ^{86}Rb uptake measured in the presence of 2.5×10^{-3} M ouabain. Results are expressed as fractional change (with respect to control value, i.e., absence of ouabain) and are means ± SE from three or four independent experiments. (A) The ouabain sensitivity of the endogenous Na,K-ATPase of nontransfected (NT) COS-7 cells was monophasic ($IC_{50} = 2 \times 10^{-7}$ M) and best described by Eq. 1 ($r^2 = 0.97$; see MATERIALS AND METHODS). (B–D) COS-7 cells transfected with the wild-type (B; WT), T15A/S16A mutant (C), or S16D mutant (D) *Bufo* α1 subunit cDNAs exhibited a bimodal ouabain inhibition pattern best described by Eq. 2 (r^2 = 0.95–0.98; see MATERIALS AND METHODS). The Na,K-ATPase populations with high ouabain sensitivity exhibited IC_{50} values between 2×10^{-8} and 4×10^{-7} M, and the Na, K-ATPase populations with lower ouabain sensitivity displayed IC_{50} values between 1 and 3.5×10^{-5} M.

Kinetic parameters were determined by nonlinear regression analysis using Prism 2.0 software. $K_{0.5Na}$ values were compared by Student's *t* test for unpaired data.

RESULTS

Functional Expression of Ouabain-resistant Na,K-ATPase in Stably Transfected COS-7 Cells

The expression of functional ouabain-resistant *Bufo* Na,K-ATPase in transfected COS-7 cells was revealed by the dose–inhibition curve of ^{86}Rb uptake by ouabain (from 10^{-8} to 2.5×10^{-3} M). Figure 1A shows that nontransfected COS-7 cells (NT) exhibit a monophasic inhibition pattern consistent with the presence of a single population of endogenous Na,K-ATPase units. In contrast, COS-7 cells expressing wild-type (WT, Figure 1B) *Bufo* α1 subunits, T15A/S16A α-mutants (Figure 1C), or S16D α-mutants (Figure 1D) exhibited a bimodal inhibition pattern. The IC_{50} of the Na,K-ATPase populations with high ouabain sensitivity (2×10^{-8}–4×10^{-7} M) and that with low ouabain sensitivity (1–3.5×10^{-5} M) reasonably fit with the IC_{50} of endogenous COS-7 cell Na,K pumps (Vilsen, 1992) and *Bufo* Na,K pumps (Jaisser *et al.*, 1993), respectively. These results show that the endogenous β subunits of COS-7 cells associate with exogenous *Bufo* wild-type or mutant α1 subunits to form functional ouabain-resistant α–β complexes (hybrid pumps), which account for the 40–60% residual ^{86}Rb uptake measured in the presence of 2.5×10^{-6} M ouabain (Figure 1, B–D).

In all subsequent experiments, the exogenous Na,K-ATPase-mediated ^{86}Rb uptake was measured in the presence of 2.5×10^{-6} M ouabain during preincubation of cells as well as incubation with ^{86}Rb.

Effect of PDBu on the Transport Activity of Na,K-ATPase Is Temperature Dependent

We first determined the effect of the activation of phorbol ester-sensitive PKCs by PDBu on the transport activity of endogenous Na,K-ATPase in nontransfected COS-7 cells and of exogenous Na,K-ATPase in COS-7 cells stably transfected with the wild-type *Bufo* α1 subunit. As depicted in Figure 2, 10^{-7} M PDBu for 15 min at 37°C inhibited the endogenous Na,K-ATPase-mediated ^{86}Rb uptake by 46 ± 6% ($p < 0.01$) and the exogenous Na,K-ATPase-mediated ^{86}Rb uptake by 30 ± 5% ($p < 0.01$) in nontransfected cells and in cells expressing the wild-type *Bufo* α1 subunit, respectively. In contrast, when temperature was lowered to 18°C, 10^{-7} M PDBu for 30 min stimulated the Na,K-ATPase-mediated ^{86}Rb uptake by 29 ± 13% ($p < 0.02$) in nontransfected cells and by 26 ± 9.5% ($p < 0.03$) in cells expressing the wild-type *Bufo* α1 subunit.

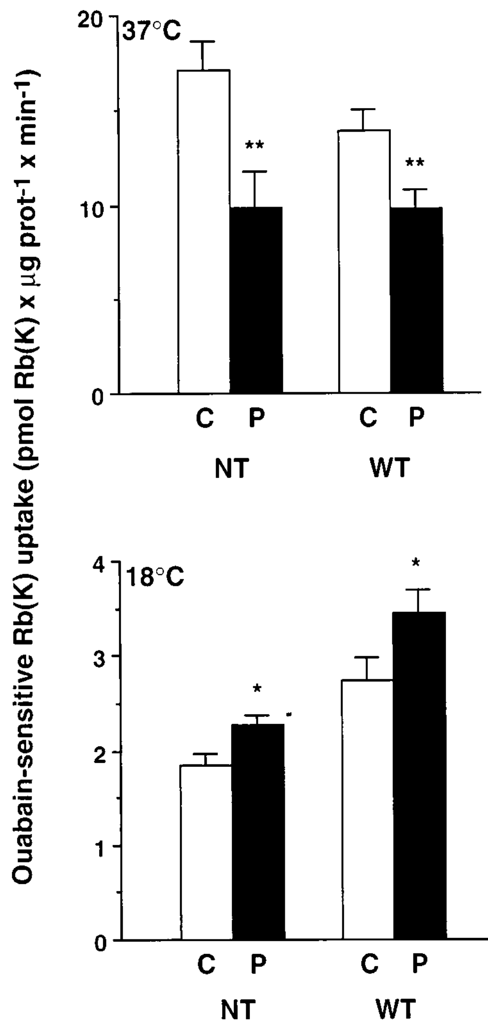

Figure 2. PDBu inhibits or stimulates the transport activity of Na, K-ATPase in COS-7 cells incubated at 37 and 18°C, respectively. Na,K-ATPase-mediated ^{86}Rb uptake was measured under initial rates of influx in nontransfected (NT) COS-7 cells or in cells stably expressing the wild-type (WT) *Bufo* α1 subunit. In transfected cells, ^{86}Rb uptake was determined in the presence of 2.5×10^{-6} M ouabain, which completely inhibits the endogenous Na, K-ATPase. Cells were incubated without (C, open bars) or with 10^{-7} M PDBu (P, filled bars) for 15 or 30 min in experiments performed at 37°C (upper panel) and 18°C (lower panel), respectively. Results are expressed as picomoles of Rb (K) $\times$ minute^{-1} $\times$ microgram of protein^{-1} and are means $\pm$ SE from 7–20 independent experiments (*, $p < 0.05$; **, $p < 0.01$).

At 37°C, PDBu decreased the ouabain-insensitive ^{86}Rb uptake (as pmol of Rb [K] $\times$ μg of protein^{-1} $\times$ min^{-1} $\pm$ SE) from 6.27 $\pm$ 0.65 to 4.33 $\pm$ 0.51 ($p < 0.05$) in nontransfected cells and from 10.08 $\pm$ 0.81 to 7.86 $\pm$ 0.53 ($p < 0.05$) in transfected cells. In contrast, at 18°C PDBu did not alter the ouabain-insensitive ^{86}Rb uptake both in nontransfected (control, 1.47 $\pm$ 0.06; PDBu, 1.60 $\pm$ 0.07) and transfected (control, 2.75 $\pm$ 0.19; PDBu, 2.56 $\pm$ 0.18) cells.

Thus, activation of phorbol ester-sensitive PKCs inhibits or stimulates the transport activity of Na,K-ATPase according to the incubation temperature.

In COS-7 Cells Incubated at 37°C, PDBu Down-regulates Na,K-ATPase Independently of the Phosphorylation of the α1 Subunit at Ser-16

Using the amphibian A6 epithelial cell line, Beron *et al.* (1997) have reported that activation of phorbol ester-sensitive PKCs induces a Ser-16 phosphorylation-independent down-regulation of cell surface Na,K-ATPase through an increase in fluid phase endocytosis. The following experiments were performed to determine whether a similar mechanism operates in mammalian cells.

As shown in Figure 3A, in COS-7 cells expressing either the wild-type *Bufo* α1 subunit (left panel) or its S943A α1-mutant, i.e., the protein kinase A (PKA) phosphorylation site mutant (our unpublished results), 10^{-7} M PDBu for 15 min at 37°C inhibited the exogenous Na,K-ATPase-mediated ^{86}Rb uptake by 30 $\pm$ 5% ($p < 0.01$) and 31 $\pm$ 7% ($p < 0.05$), respectively. In COS-7 cells expressing the T15A/S16A α1-mutant in which the PKC-dependent phosphorylation is abolished (Béguin *et al.*, 1994, 1996a), PDBu still inhibited the exogenous Na,K-ATPase-mediated ^{86}Rb uptake by 19 $\pm$ 3% ($p < 0.05$; Figure 3A, middle panel). Finally, the ouabain-sensitive ^{86}Rb uptake was inhibited by 33 $\pm$ 8% ($p < 0.05$; Figure 3A, right panel) by PDBu in cells expressing the S16D α1-mutant, which contains a negative charge mimicking constitutive phosphorylation. Similar results were obtained in cells expressing the S16E α1-mutant (our unpublished results).

The effect of PDBu on the cell surface expression of the exogenous Na,K-ATPase was estimated using a biotinylation-streptavidin precipitation assay. Figure 3B shows that 10^{-7} M PDBu for 15 min at 37°C decreased the number of biotinylated, cell surface–located, wild-type *Bufo* α1 subunits and T15A/S16A and S16D α1-mutants by 25 $\pm$ 6, 18 $\pm$ 5, and 29 $\pm$ 9%, respectively.

These results demonstrate that, like in amphibian cells (Beron *et al.*, 1997), PDBu down-regulates the cell surface Na,K-ATPase independently of the phosphorylation of its catalytic α1 subunit at Ser-16 in the mammalian COS-7 cell line.

The Inhibition of the Transport Activity of Na,K-ATPase by PDBu at 37°C is Dependent on PKC and Arachidonic Acid Metabolism

To evaluate the specificity of the effects of PDBu, the ouabain-sensitive ^{86}Rb uptake and the cell surface expression of Na,K-ATPase were studied under conditions in which PKC was inhibited. Preincubation of cells for 15 min at 37°C in the presence of 5×10^{-7} M GF109203X, a specific PKC inhibitor (Toullec *et al.*, 1991), prevented both the PDBu-induced inhibition of the transport activity of the exogenous Na,K-ATPase (Figure 4A) and its decrease in cell surface expression (Figure 4B) in cells expressing the wild-type *Bufo* α1 subunit. Similar results were obtained in cells expressing the T15A/S16A α1-mutant (our unpublished results). These observations indicate that activation of phorbol ester-sensitive PKC(s) mediates the effects of PDBu.

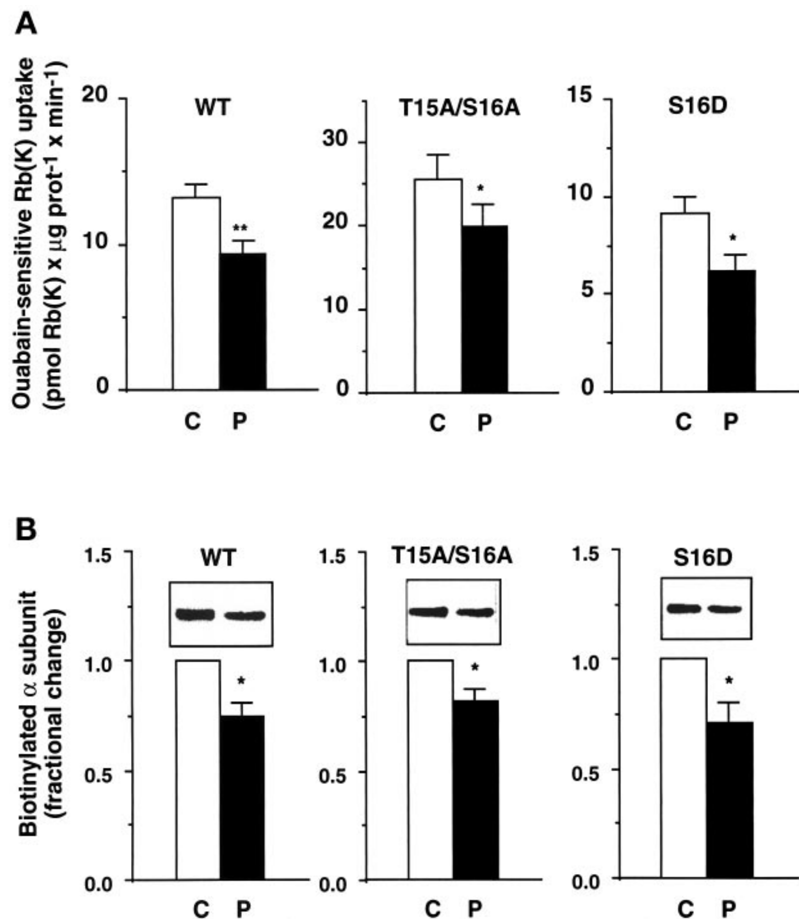

Figure 3. In COS-7 cells incubated at 37°C, the effects of PDBu on the transport activity and the cell surface expression of Na, K-ATPase are independent of the phosphorylation of the α1 subunit at Ser-16. COS-7 cells stably expressing the wild-type (WT) *Bufo* α1 subunit, the T15A/S16A mutant, or the S16D mutant mimicking constitutive phosphorylation were incubated for 15 min at 37°C in the absence (C, open bars) or presence of 10^{-7} M PDBu (P, filled bars). (A) Exogenous Na,K-ATPase-mediated ^{86}Rb uptake was measured in the presence of 2.5×10^{-6} M ouabain under initial rates of influx. Results are expressed as picomoles of Rb (K) × minute^{-1} × microgram of protein and are means ± SE from 7–12 independent experiments (*, $p < 0.05$; **, $p < 0.01$). (B) The effect of PDBu on the cell surface expression of *Bufo* α1 subunits was measured by Western blot with anti-*Bufo* α1 subunit antibody after streptavidin precipitation of the biotinylated cell surface proteins. Insets show a representative experiment, and bars show the quantitation of the relative amounts of cell surface Na,K-ATPase α1 subunits. Results are expressed as fractional change (with respect to control value) and are means ± SE from 6–18 independent experiments (*, $p < 0.05$).

In various cell types, the inhibitory effect of phorbol esters on Na,K-ATPase activity rely on a PKC-mediated phospholipase A_2 (PLA_2) activation (Satoh *et al.*, 1993b; Xia *et al.*, 1995) and arachidonic acid metabolism through the cytochrome P450-dependent monooxygenase pathway (Schwartzman *et al.*, 1985; Satoh *et al.*, 1992, 1993a; Chibalin *et al.*, 1998). Therefore, we evaluated the role of this pathway in the down-regulation of Na,K-ATPase by PDBu in COS-7 cells expressing the wild-type *Bufo* α1 subunit. Preincubation for 15 min at 37°C with 5×10^{-6} M arachidonic acid or 10^{-7} M PDBu decreased the transport activity (Figure 4A) and the cell surface expression (Figure 4B) of the exogenous Na,K-ATPase to the same extent. On the other hand, the inhibition of PLA_2 by 15 min of preincubation at 37°C with 10^{-5} M oleoyloxyethylphosphocholine prevented the PDBu-induced inhibition of the ouabain-sensitive ^{86}Rb uptake (Figure 4A) and the decrease in cell surface expression of Na,K-ATPase (Figure 4B). Similarly, 10^{-5} M mepacrine, another PLA_2 inhibitor, abolished the effect of PDBu on the transport activity of Na,K-ATPase (as pmol of Rb [K] × µg of protein^{-1} × min^{-1}: control, 13.2 ± 0.8; PDBu, 9.3 ± 1.0; mepacrine, 12.0 ± 0.8; mepacrine and PDBu, 12.6 ± 0.6). In agreement with these observations, the inhibition of the cytochrome P450-dependent monooxygenase pathway by 15 min of preincubation at 37°C with 10^{-6} M 17-octadecynoic acid abrogated the effects of PDBu on the transport activity (Figure 4A) and the cell surface expression (Figure 4B) of Na,K-ATPase. In agreement with this result, 10^{-6} M SKF525A, another structurally unrelated inhibitor of the cytochrome P450-dependent monooxygenase, prevented the PDBu-dependent inhibition of ouabain-sensitive ^{86}Rb uptake (as pmol of Rb [K] × µg of protein^{-1} × min^{-1}; control, 12.5 ± 1.2; PDBu, 8.6 ± 1.3; SKF, 11.4 ± 1.0; SKF and PDBu, 10.3 ± 1.3).

Altogether, these results may suggest that the PKC-mediated down-regulation of Na,K-ATPase is dependent on PLA_2 activation and the subsequent generation of arachidonic acid metabolites through the cytochrome P450-dependent monooxygenase pathway in COS-7 cells.

The Stimulation of the Transport Activity of Na,K-ATPase by PDBu at 18°C Is Dependent on Phosphorylation of the α1 Subunit at Ser-16

As shown in Figure 2, lowering the incubation temperature to 18°C revealed a stimulatory effect of PDBu on the ouabain-sensitive ^{86}Rb uptake. The following experiments were therefore performed to determine the role of phosphorylation of the α1 subunit at Ser-16 in the PDBu-induced stimulation of the transport activity of Na,K-ATPase at 18°C. In COS-7 cells expressing either the wild-type *Bufo* α1 subunit (Figure 5A) or its S943A α1-mutant, i.e., the PKA phos-

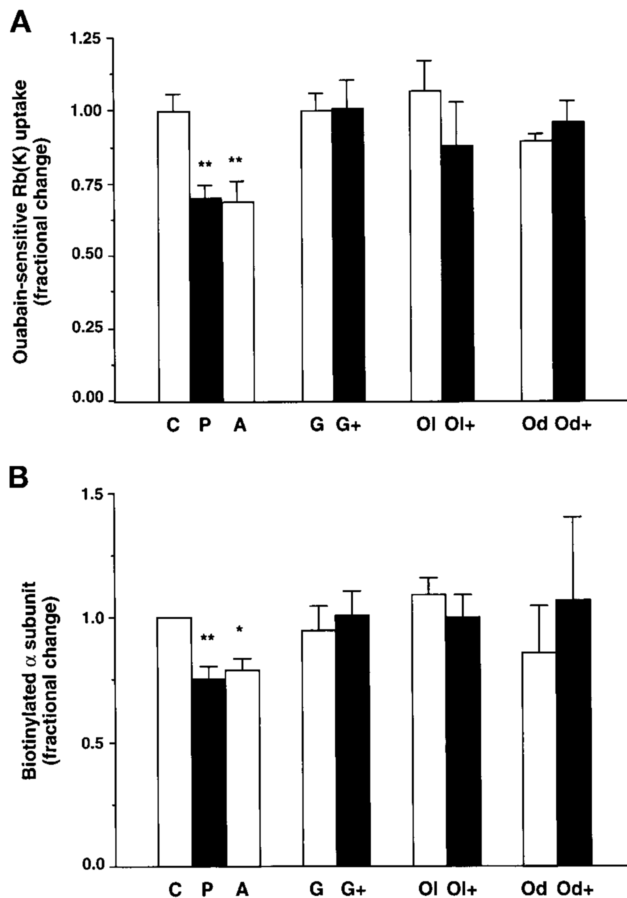

Figure 4. In COS-7 cells incubated at 37°C, the effects of PDBu on the transport activity and the cell surface expression of Na,K-ATPase are dependent on PKCs and arachidonic acid metabolism. COS-7 cells expressing the wild-type *Bufo* α1 subunit were incubated for 30 min at 37°C in the absence (C) or presence of 10^{-7} M PDBu (P), 5×10^{-6} M arachidonic acid (A), 5×10^{-7} M GF109203X (a PKC inhibitor; G), GF109203X and PDBu (G+), 10^{-5} M oleoyloxyethylphosphocholine (a phospholipase A_2 inhibitor; Ol), oleoyloxyethylphosphocholine and PDBu (Ol+); 17-octadecynoic acid (a cytochrome P450 inhibitor; Od), and 17-octadecynoic acid and PDBu (Od+). (A) Exogenous Na,K-ATPase-mediated ^{86}Rb uptake was measured in the presence of 2.5×10^{-6} M ouabain under initial rates of influx. Results are expressed as fractional change (with respect to control value) and are means ± SE from 6–14 independent experiments (**, $p < 0.01$). (B) After streptavidin precipitation, the cell surface–expressed *Bufo* α1 subunits were detected by immunoblotting using a specific anti-*Bufo* α1 subunit antibody. Results are expressed as fractional change (with respect to control values) and are means ± SE from six or seven independent experiments (*, $p < 0.05$).

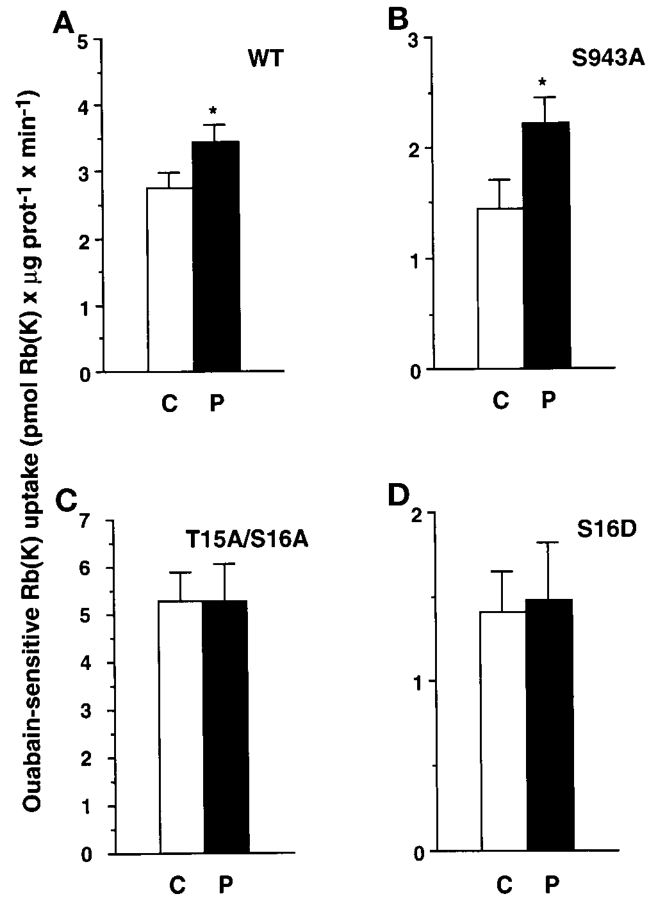

Figure 5. In COS-7 cells incubated at 18°C, the PDBu-induced stimulation of the transport activity of the Na,K-ATPase is abolished by mutation of the α1 subunit at Ser-16. Exogenous Na,K-ATPase-mediated ^{86}Rb uptake was measured in the presence of 2.5×10^{-6} M ouabain under initial rates of influx in COS-7 cells stably expressing the wild-type (A, WT) *Bufo* α1 subunit, the S943A (PKA site) mutant (B), the T15A/S16A mutant (C), or the S16D mutant mimicking constitutive phosphorylation (D). Cells were incubated for 30 min at 18°C in the absence (C, open bars) or in the presence of 10^{-7} M PDBu (P, filled bars). Results are expressed as picomoles of Rb (K) × microgram of protein^{-1} × minute^{-1} and are means ± SE from 7–20 independent experiments (*, $p < 0.05$).

phorylation site mutant (Figure 5B), 10^{-7} M PDBu for 30 min at 18°C stimulated the exogenous Na,K-ATPase-mediated ^{86}Rb uptake by 26 ± 9% ($p < 0.05$) and 53 ± 11% ($p < 0.05$), respectively. The PDBu-induced stimulation of the exogenous Na,K-ATPase-mediated ^{86}Rb uptake was abolished in COS-7 cells that expressed the T15A/S16A α1-mutant (Figure 5C), which is no longer phosphorylated by PKC (Béguin *et al.*, 1994, 1996a). Finally, the ouabain-sensitive ^{86}Rb uptake was not altered by PDBu in cells expressing the S16D α1-mutant (Figure 5D), which contains a negative charge and thus mimics constitutive phosphorylation. Altogether, these results strongly suggest that the stimulation of the ouabain-sensitive ^{86}Rb uptake by PDBu observed at 18°C relies on phosphorylation of the catalytic α1 subunit of Na,K-ATPase at Ser-16.

PDBu Does Not Change the Cell Surface Expression of Na,K-ATPase in Cells Incubated at 18°C

Biotinylation assays were performed on intact COS-7 cells expressing wild-type *Bufo* α1 subunits. Figure 6, A and B, shows that neither 10^{-7} M PDBu nor 5×10^{-7} M GF109203X, a specific PKC inhibitor, for 30 min at 18°C

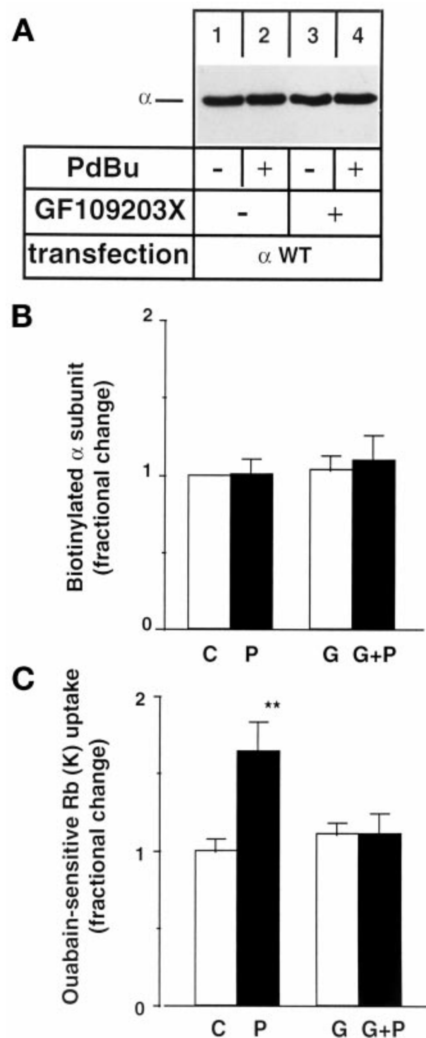

Figure 6. Incubation at 18°C prevents the PDBu-induced down-regulation of cell surface Na,K-ATPase. COS-7 cells expressing wild-type *Bufo* α1 subunit were preincubated for 30 min at 18°C in the absence or in the presence of 10^{-7} M PDBu (P), 5×10^{-7} M GF109203X (G), or PDBu and GF109203X (G+P) before biotinylation of cell surface proteins (A and B) or measurement of exogenous Na,K-ATPase-mediated ^{86}Rb uptake (C). After streptavidin precipitation, the cell surface–expressed *Bufo* α1 subunit was detected by immunoblot using a specific anti-*Bufo* α1 subunit antibody. (A) A representative immunoblot is shown (n = 4). (B) Quantitation of the relative amounts of Na,K-ATPase α1 subunit detected by immunoblotting. Results are expressed as fractional change (with respect to control value) and are means ± SE from four independent experiments. (C) Ouabain-sensitive ^{86}Rb uptake. Results are expressed as fractional change (with respect to control value) and are means ± SE from 11 independent experiments (**, $p < 0.01$).

changed the cell surface expression of exogenous Na,K pumps. In contrast, the PDBu-induced stimulation of the exogenous Na,K-ATPase-mediated ^{86}Rb transport was abolished by GF109203X (Figure 6C).

This result shows that the PKC phosphorylation-dependent stimulation of the transport activity of Na,K-ATPase observed at 18°C is not due to an increase in the number of active Na,K-pumps located at the cell surface.

α1-Mutants Mimicking Ser-16 Phosphorylation Increase the Apparent Na Affinity of Na,K-ATPase

The absence of alteration in cell surface expression of Na,K-pumps in cells incubated with PDBu at 18°C may indicate that the Ser-16 phosphorylation-dependent stimulation of the transport activity of Na,K-ATPase could be achieved by an alteration of the functional properties of preexisting pumps.

We therefore analyzed the Na activation curves of the hydrolytic activity of the exogenous Na,K-ATPase in crude membranes from transfected COS-7 cells expressing the wild-type *Bufo* α1 subunit or its S16D and S16E mutants. The results show that compared with wild-type α1 subunits, expression of S16D (Figure 7A) and S16E (our unpublished results) α-mutants induced a significant leftward shift of the Na activation curve of the hydrolytic activity of the exogenous Na,K-ATPase. This finding reflects an increase in the apparent Na affinity of the Na,K pumps containing the constitutive phosphorylation mutant α subunits ($K_{0.5Na}$ [mM ± SE]: wild type, 6.72 ± 0.63; S16D, 2.34 ± 0.42*; S16E, 4.82 ± 0.25*; *$p < 0.01$). Figure 7B shows that expression of the T15A/S16A α-mutant that suppresses the phosphorylation site does not alter the apparent Na affinity of the exogenous Na,K-ATPase ($K_{0.5Na}$, 6.32 ± 0.68). This observation supports the notion of a specific effect of negatively charged residues that mimic the effect of phosphorylation. These results strongly suggest that phosphorylation of the Na,K-ATPase α1 subunit on Ser-16 induces an increase in the apparent Na affinity of the enzyme, which can account for the stimulation of the cation transport activity of Na,K-ATPase in response to phorbol ester-sensitive PKC(s) activation in intact cells.

PDBu Increases the Na Sensitivity of the Hydrolytic Activity of Na,K-ATPase in Permeabilized COS-7 Cells

The following experiments were designed to assess whether in addition to the down-regulation of cell surface Na,K-ATPase, PDBu also increases the apparent Na affinity of the enzyme in nontransfected COS-7 cells preincubated at 37°C. After 15 min of preincubation at 37°C in the absence or the presence of 10^{-7} M PDBu, cells were permeabilized, and the hydrolytic activity of Na,K-ATPase was measured in the presence of increasing concentrations of Na (from 0 to 70 mM). In agreement with the down-regulation of cell surface Na,K-pumps (see Figure 3B), PDBu inhibited the maximal hydrolytic activity of Na,K-ATPase measured in the presence of a saturating Na concentration (as pmol of ATP × μg of protein^{-1} × h^{-1}: control, 484 ± 26; PDBu, 348 ± 34; $p < 0.005$). However, Figure 8 shows that PDBu also increased the Na sensitivity of the Na,K-ATPase, as shown by the leftward shift of the Na activation curve. Indeed, the maximal Na,K-ATPase activity is reached in the presence of 15 and 50 mM Na in control and PDBu-treated cells, respectively. Therefore, PDBu decreases the maximal activity and increases the Na sensitivity of Na,K-ATPase in COS-7 cells incubated at 37°C.

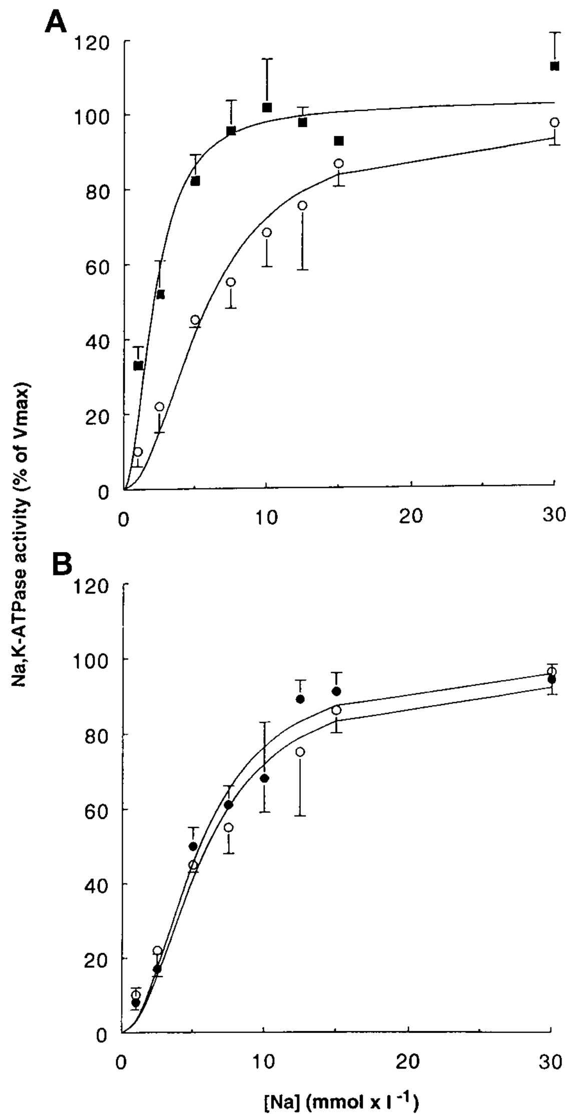

Figure 7. A mutant mimicking phosphorylation of the α1 subunit at Ser-16 (S16D) increases the apparent Na affinity of Na,K-ATPase. The Na activation of the hydrolytic activity of the hybrid Na,K pumps was measured in permeabilized membranes isolated from stably transfected COS-7 cells. Results are expressed as the percentage of maximal activity and are means ± SE from four to seven independent experiments. (A) Na activation curves of Na,K-ATPase from cells expressing the wild-type *Bufo* α1 subunit (open circles) or the S16D α-mutant (closed squares). (B) Na activation curves of Na,K-ATPase from cells expressing the wild-type *Bufo* α1 subunit (open circles) or the T15A/S16A α-mutant (closed circles). The activity of exogenous Na,K-ATPase measured in the presence of 30 mM Na was 586 ± 9 (n = 6), 314 ± 47 (n = 4), and 631 ± 85 (n = 5) pmol of ATP × h^{-1} × μg of protein^{-1} for the wild-type, the S16D mutant, and the T15A/S16A mutant Na,K-pumps, respectively.

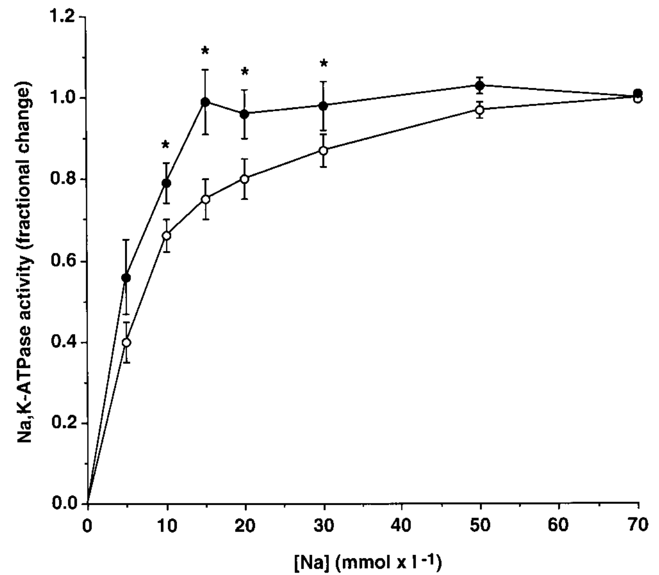

Figure 8. PDBu increases the Na sensitivity of Na,K-ATPase in permeabilized COS-7 cells. After preincubation in the absence (C, open bars) or presence of 10^{-7} M PDBu (P, filled bars) at 37°C, COS-7 cells were permeabilized by freeze–thawing. The hydrolytic activity of Na,K-ATPase was measured in permeabilized cells in the presence of increasing Na concentrations (from 0 to 70 mM). Results are expressed as a fraction of the maximal Na,K-ATPase activity and are means ± SE from 12 independent experiments (* $p < 0.05$). The maximal Na,K-ATPase activity was 484 ± 26 and 348 ± 34 pmol of ATP × μg of protein^{-1} × h^{-1} ± SE for control and PDBu-treated cells, respectively.

DISCUSSION

Expression of an ouabain-resistant wild-type Na,K-ATPase α1 subunit or its T15A/S16A and S16D mutants in COS-7 cells permitted us to inhibit the endogenous Na,K pumps and to study the direct relationship existing between α1 subunit Ser-16 phosphorylation and modulation of Na,K-ATPase activity. The present study provides evidence that in addition to the previously described Ser-16 phosphorylation-independent down-regulation of cell surface Na,K-ATPase (Beron *et al.*, 1997), the activation of phorbol ester-sensitive PKC(s) induces a Ser-16 phosphorylation-dependent increase in the apparent Na affinity of Na,K-ATPase.

In agreement with the findings of Beron *et al.* (1997) in A6 epithelial cells, our study shows that the inhibition Na,K-ATPase by phorbol esters in COS-7 cells incubated at 37°C relies on a down-regulation of cell surface Na,K-pumps and is independent of Ser-16 phosphorylation (see Figure 3). Indeed, these effects of phorbol esters are not altered in cells expressing the T15A/S16A α-mutant, which is not phosphorylated. Furthermore, expression of an α-mutant in which Ser-16 is substituted by a negatively charged amino acid (Asp or Glu) mimicking permanent phosphorylation of the α subunit did not prevent either the inhibition of Na,K-ATPase or its decrease in cell surface expression in response to PDBu.

The decrease in activity and cell surface expression of Na,K-ATPase observed after phorbol ester treatment at 37°C is likely to be mediated by a PKC-dependent PLA_2 activation and subsequent metabolism of the generated free arachidonic acid through the cytochrome P-450-dependent monooxygenase (CP-450) pathway. Indeed, these effects are

prevented by the inhibition of PKC, of PLA_2, and of CP-450 (see Figure 4). In addition, the effects of PDBu are mimicked by arachidonic acid. Several lines of evidence support the physiological importance of this observation: 1) PKC can directly phosphorylate and activate PLA_2 (Nemenoff *et al.*, 1993); and 2) in various cells, including renal epithelial cells, the PKC-dependent inhibition of Na,K-ATPase activity relies on PLA_2 activation and arachidonic metabolism through CP-450 (Schwartzman *et al.*, 1985; Satoh *et al.*, 1993b; Xia *et al.*, 1995).

The Ser-16 phosphorylation-dependent stimulation of the transport activity of a representative α1-β Na,K-ATPase isozyme complex demonstrated in COS-7 cells incubated at 18°C (see Figure 5) is most likely mediated by a change in its apparent Na affinity. Indeed, in permeabilized COS-7 cells, PDBu increases the Na sensitivity of Na,K-ATPase, and this effect is large enough to fully account for the PDBu-induced increase in ouabain-sensitive Rb (K) uptake measured in intact cells (see Figure 8). Because under these experimental conditions the transmembrane ion gradients are abolished and the intracellular and extracellular Na concentrations are equal and constant, the latter observation implies that PDBu increased the apparent Na affinity of the fraction of Na,K pumps that remained active at the cell surface. The present results confirm the previously described increase in the apparent Na affinity of Na,K-ATPase in response to PKC activation in isolated proximal convoluted tubules (Féraille *et al.*, 1995). The PDBu-induced increase in apparent Na affinity of Na,K-ATPase most likely relies on Ser-16 phosphorylation, because this effect is reproduced by α1-mutants mimicking constitutive Ser-16 phosphorylation (see Figure 7A). This effect of Ser-16 phosphorylation on the apparent Na affinity of Na,K-ATPase is in agreement with the results of Logvinenko *et al.* (1996), who showed that in vitro phosphorylation of purified Na,K-ATPase by PKC shifts the conformational equilibrium of the Na,K pump toward E1, i.e., the Na conformation. These observations are consistent with earlier studies showing that the α1 subunit NH_2-terminal domain is involved in conformational changes of the enzyme. Indeed, tryptic cleavage of the α1 subunit in the E1 conformation occurring between Lys-30 and Glu-31 (Jorgensen and Collins, 1986) or truncations of the NH_2 terminus by site-directed mutagenesis (Wierzbicki and Blostein, 1993; Wang *et al.*, 1996) displace the E1–E2 conformational equilibrium in direction of the E1 conformation through an increased rate of potassium deocclusion (Wierzbicki and Blostein, 1993), which may account for the increased apparent Na affinity of Na,K-ATPase (Jorgensen andCollins, 1986).

Altogether, our results indicate that the down-regulation of cell surface Na,K-ATPase in response to phorbol esters masks the intrinsic functional effect of Ser-16 phosphorylation of the Na,K-ATPase α subunit in COS-7 cells incubated at 37°C. This observation is in agreement with a growing number of studies, which report stimulation of Na,K-ATPase activity in response to PKC activation (Lynch *et al.*, 1986; Hootman *et al.*, 1987; Gupta *et al.*, 1991; Féraille *et al.*, 1995; Pedemonte *et al.*, 1997) but contrasts with findings on rat α1 subunits in which PKC-dependent phosphorylation inhibits (Belusa *et al.*, 1997) or does not alter (Feschenko and Sweadner, 1997) Na,K-ATPase activity. However, our study and these former studies (Belusa *et al.*, 1997; Feschenko and Sweadner, 1997) cannot be directly compared, because we specifically studied the role of the ubiquitous Ser-16 phosphorylation site, whereas others focused on the role of the rat-specific Ser-23 phosphorylation site. Indeed, among higher vertebrates, the rat α1 subunit is the only one that exhibits two PKC phosphorylation sites: the ubiquitous site on Ser-16 and an additional site on Ser-23 (Feschenko and Sweadner, 1995; Béguin *et al.*, 1996b), accounting for 80% of in vitro PKC phosphorylation in this species (Feschenko and Sweadner, 1995). These two phosphorylation sites might be targets for different PKC isozymes and/or produce different physiological effects. Ser-23 is indeed located within a consensus PKC site lying within the lysine cluster, whereas Ser-16 is part of a novel unconventional PKC phosphorylation site (Béguin *et al.*, 1996b). This hypothesis is indirectly supported by data from Vasilets (1997), which show that the transport activity of rat α1–β complexes expressed in *Xenopus* oocytes is inhibited, whereas that of the endogenous, *Xenopus* α1–β complexes, which were previously shown to be exclusively phosphorylated on Ser-16 (Béguin *et al.*, 1996b), are stimulated by injection of purified rat PKC.

In conclusion, our results show that phosphorylation of the α subunit of Na,K-ATPase on Ser-16 may stimulate its activity through an increase in apparent Na affinity. Thus, phosphorylation of the Na,K-ATPase α1 subunit on Ser-16 in response to the activation of phorbol ester-sensitive PKC(s) is likely to play a critical role in the homeostasis of intracellular monovalent cation concentration as well as in repolarization of excitable cells and vectorial ion transport by epithelial cells. In addition, the present study provides evidence that in some cells, the activity of Na,K-ATPase can be controlled by an additional mechanism, which alters membrane trafficking and changes the cell surface expression of Na,K pumps independently of Ser-16 phosphorylation of the α1 subunit.

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