Dopamine-induced Endocytosis of Na\textsuperscript{+},K\textsuperscript{+}-ATPase Is Initiated by Phosphorylation of Ser-18 in the Rat α Subunit and Is Responsible for the Decreased Activity in Epithelial Cells*

(Received for publication, April 27, 1998, and in revised form, September 25, 1998)

Alexander V. Chibalin, Goichi Ogimoto, Carlos H. Pedemonte, Thomas A. Pressley, Adrian I. Katz, Eric Féralle, Per-Olof Berggren, and Alejandro M. Bertorello**

From the Department of Molecular Medicine, Karolinska Institutet, The Rolf Luft Center for Diabetes Research, Karolinska Hospital, S-171 76 Stockholm, Sweden, the §Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas 77204, the ¶Department of Physiology, Texas Tech University, Lubbock, Texas 79430, the DePARTMENT of Medicine, University of Chicago, Chicago, Illinois 60637, and the Division de Néphrologie, Hôpital Cantonal Universitaire, CH-1211 Geneva 14, Switzerland

Dopamine inhibits Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in renal tubule cells. This inhibition is associated with phosphorylation and internalization of the α subunit, both events being protein kinase C-dependent. Studies of purified preparations, fusion proteins with site-directed mutagenesis, and heterologous expression systems have identified two major protein kinase C phosphorylation events being protein kinase C-dependent. Studies of port proteins: while Na\textsuperscript{+} requires endocytosis of the Ser-18 is essential for this process.

We conclude that dopamine-induced inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in rat renal tubule cells requires endocytosis of the α subunit into defined intracellular compartments and that phosphorylation of Ser-18 is essential for this process.

Regulation of the Na\textsuperscript{+} channel (1), H\textsuperscript{+},K\textsuperscript{+}-ATPase (2) and Na\textsuperscript{+},K\textsuperscript{+}-ATPase (3, 4) activity appears to involve removal from, or retention within, the plasma membrane of these transport proteins: while Na\textsuperscript{+} channels and H\textsuperscript{+},K\textsuperscript{+}-ATPase activity in intact cells are constitutively increased by the removal of specific endocytic signals, internalization of Na\textsuperscript{+},K\textsuperscript{+}-ATPase is associated with activation of specific membrane receptors and depends on the sequence of intracellular signals generated from this interaction (4, 5).

Endocytosis of integral membrane proteins is initiated by selective recognition of the target protein followed by its transport, usually via clathrin-coated vesicles (CCV), into endosomes (6–9). While endocytosis of membrane receptors as part of their signaling mechanism has been extensively studied, internalization of integral membrane proteins triggered by a network of signals generated after activation of G protein-coupled receptors (GPCRs) is less well documented.

Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by activation of GPCRs (dopaminergic) in renal epithelial cells is associated with the removal of active units from the basolateral plasma membrane and their transport, via clathrin-coated vesicles, into early (EE) and late endosomes (LE) (4). This process requires activation of phosphatidylinositol 3-kinase (10) and is dependent on protein kinase C (11). Protein kinase C (PKC) phosphorylates the Na\textsuperscript{+},K\textsuperscript{+}-ATPase α1 subunit in cell-free preparations (12–16) and this event is associated with a decrease in its catalytic activity (13, 15). However, because in some reports investigators have failed to demonstrate an effect of PKC on the hydrolytic activity of purified preparations (16, 17), or of phorbol esters in cell lines (18), it remains unclear whether Ser/Thr phosphorylation of the α1 subunit represents a physiological mechanism responsible for Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibition in intact cells.

In PCT (proximal convoluted tubule) segments phorbol esters (19, 20) and diacylglycerol analogs (20, 21) inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. In OK (opossum kidney) cells and Xenopus oocytes phorbol esters decrease ouabain-sensitive Rb\textsuperscript{+} transport, and this decrease is associated with phosphorylation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase α1 subunit (22, 23). However, expression of a mutated form (Ser-18) alone or in combination with another mutation (Ser-11) in Xenopus oocytes revealed a variable response of Rb\textsuperscript{+} transport, depending on whether phorbol esters or purified PKC was used (23). Moreover, phorbol esters have been also associated with stimulation of ouabain-sensitive Rb\textsuperscript{+} transport in PCT segments (24, 25) and OK cells (26).

Earlier experimental approaches often relied on the action of phorbol esters as direct activators of protein kinase C. The variety of Na\textsuperscript{+},K\textsuperscript{+}-ATPase responses to these agents seen in

* This work was supported in part by funds from the Swedish Medical Research Council (to A. M. B., and P.-O. B), the National Institutes of Health (to C. H. P. (Grant DK52273) and T. A. P.), FNRS (to E. F.), and from the American Heart Association (to C. H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: The Rolf Luft Center L6B:01, Karolinska Hospital, S-171 76 Stockholm, Sweden. Tel.: 46-8-517-75727; Fax: 46-8-517-73658; E-mail: alejan@enk.ki.se.

† The abbreviations used are: CCV, clathrin-coated vesicles; GPCRs, G protein-coupled receptors; PKC, protein kinase C; PCT, proximal convoluted tubules; OK, opossum kidney; Bis, bisindolylmaleimide; DA, dopamine; Wt, wild type; EE, early endosomes; LE, late endosomes; MES, 2-(N-morpholino)ethanesulfonic acid.
intact cells could represent compensatory homeostatic mechanisms due to phosphorylation of numerous substrates, resulting for example, in increased fluid phase endocytosis (27) that would affect Na,K-ATPase function. Using a physiologic ligand (dopamine) we have demonstrated that inhibition of Na,K-ATPase activity in renal proximal tubule cells was associated with phosphorylation and selective endocytosis of the α subunit (4, 11). Furthermore, these two events were connected, because the rat α subunit carrying an amino-terminal deletion (first 28 residues) that includes the PKC target sites (Ser-11/Ser-18) was not internalized in response to dopamine (11).

While these results indicate that phosphorylation of the α subunit in response to dopamine is necessary for internalization, it remains unclear whether inhibition of the enzymatic activity in intact cells results from phosphorylation of the subunit in the plasma membrane (so that Na,K-ATPase undergoes a conformational change in situ that decreases its activity) or from the endocytic process, i.e. inhibition occurs only as a result of decreased Na,K-ATPase units in the plasma membrane. In addition, because the residual two residues (Ser-11 and Ser-18) phosphorylated by PKC in purified preparations (16) are within the NH₂ terminus of the α subunit, in this study we have utilized site-directed mutagenesis to determine in intact cells whether Ser-11 or Ser-18, or both are phosphorylation targets involved in Na,K-ATPase endocytosis and inhibition of its catalytic activity in response to dopamine.

**EXPERIMENTAL PROCEDURES**

**Materials—**All chemicals were from Sigma, except bisindolylmaleimide (Bis) and L2940009, which were purchased from Calbiochem. Na,K-ATPase α subunit abundance in clathrin vesicles and early and late endosomes was determined using a monoclonal antibody kindly provided by Dr. M. Caplan (Yale University). Immunoprecipitation of the Na,K-ATPase in the phosphorylation experiments was performed using a polyclonal antibody raised against the rat Na,K-ATPase α subunit (25).

**Preparation of PCT Cells—**PCT cells were prepared as described before (11). Briefly, male Sprague-Dawley rats (BK Universal, Sollen,tuna, Sweden) weighing between 150 and 200 g were used. After the kidneys were removed and the cortex isolated, the tissue was moistened on ice to a paste-like consistency. The cortical mince was incubated with 0.625 mg/ml collagenase (Type I, Sigma) in 50 ml of Hanks’ medium (Life Technologies, Inc.). The infusion was carried out at 37 °C for 60 min, the solution being continuously exposed to 95% O₂,5% CO₂ and was terminated by placing the tissue on ice and pouring through graded sieves (75–53–38 μm pore size) to obtain a cell suspension. The PCT cells were washed three to four times by centrifugation at 100 × g for 4 min in order to separate the remaining blood cells and traces of collagenase and were then kept on ice. Cells were resuspended to yield a protein concentration of approximately 3.5–5.0 mg/ml and were used immediately after preparation.

**Site-directed Mutagenesis—**Wild-type and mutant forms of the Na,K-ATPase α subunit of rat were prepared from complementary DNA for expression by mammalian cells in culture. Wild type α, cDNA was a gift of Dr. Jerry D. Lingrel (28). Wild type sequence was annealed with the oligonucleotide TATGAGCCTGAGGACGTTGGGACAAAGAACGGA and its complement. Substitution of an alanine for the second serine, Ser-11(S11A), was accomplished with the oligonucleotide TATGAGCCTGGGACGTTGGGACAAAGAACGGA and its complement. Replacement of alanine for the second serine, Ser-11(S18A), was accomplished with the oligonucleotide AGAACATGCGGAGAGGCTGTGGCGGAACATGGGGACAAGAAGA and its complement. A diagnostic HaeIII site was introduced for both mutants. Structure of the resulting mutants was evaluated by restriction with the appropriate endonuclease and confirmed byideoxynucleotide sequencing of the altered region. Mutant cDNAs were subcloned downstream of the immediate promoter of human cytomegalovirus in pcDNA3.1 (Invitrogen, San Diego, CA).

**Cell Transfection—**OK cells were transfected with the various cDNA plasmids using liposomes (26, 29). Two days after transfection, the cells were transferred to a medium containing 10 μM ouabain. Because the endogenous Na,K-ATPase of OK cells is sensitive to this level of ouabain, only OK cells that express the Na,K-ATPase containing the rodent α subunit would be able to survive. After 10 days, resistant colonies were expanded and maintained all the time in medium containing 10 μM ouabain. Experiments were performed with a mix of at least 20 clones.

**Determination of Na,K-ATPase Activity in OK Cells—**Transfected cells (maintained at all times in 10 μM ouabain) were incubated in modified Hanks’ medium in the presence or absence of 1 μM DA at room temperature for 2.5 min. The incubation was terminated by placing the samples on ice. Cell aliquots (approximately 5–15 μg of protein) were transferred to the Na,K-ATPase assay medium (final volume, 100 μl) containing in mM: 50 NaCl, 5 KCl, 10 MgCl₂, 1 EGTA, 50 Tris-HCl, 10 Na₂ATP (Calbiochem) and [γ⁻³²P]ATP (NEN Life Science Products; specific activity, 3000 Ci/mmol) in tracer amounts (3.3 nCi/μl). Na,K-ATPase activity was then determined after permeabilization of the cells as described before (4). Total Na,K-ATPase activity was measured in samples containing 10 μM ouabain. Thus, cells remained exposed to this ouabain concentration at all times, including after permeabilization, the magnesium-dependent ATPase activity was measured in the same medium containing 4 mM digitonin. The difference between these two groups represents the activity of the “transfected” ouabain-resistant isofrom.

A portion of Na,K-ATPase molecules is located in intracellular organelles. The sum of their activities (nmol of P/mg of protein/min) in CCV, ~20 and EE, ~15, constitutes approximately 30% of total cell activity, 112 nmol of P/mg of protein/min (4). Because their presence in these compartments may affect the interpretation of activities obtained in whole cells, we have validated our method by comparing the Na,K-ATPase hydrolytic activity in PCT cells permeabilized by thermic shock (~20 °C) to a well established method for cell permeabilization, e.g. treatment with digitonin (30). In five separate experiments Na,K-ATPase activity (nmol of P/mg of protein/min) in PCT cells subjected to permeabilization at ~20 °C was 99 ± 8, not significantly different from that of PCT cells permeabilized by incubation with 50 μM digitonin during 10 min at 37 °C (116 ± 13, p = 0.313). When cells previously exposed to ~20 °C for 10 min were incubated with digitonin, which now would also access the interior of the cell, the Na,K-ATPase activity was significantly increased (161 ± 9, n = 4, p < 0.05 versus digitonin alone). This increase (~38%) probably reflects to a large extent the entry of the cationic substrates in these organelles and suggests that incubation of intact cells at ~20 °C or with digitonin alone results only in permeabilization of the plasma membrane but not of organelles from intracellular organelles, such as clathrin vesicles and early endosomes.

**Phosphorylation and Immunoprecipitation of Na,K-ATPase in Intact Cells—**Rat PCT cells (4–6 mg of protein/3 ml of medium) were labeled at 32 °C in a buffer containing (in mM): 50 NaCl, 5 KCl, 4 NaHCO₃, 1 CaCl₂, 1 MgSO₄, 0.2 NaH₂PO₄, 0.15 Na₂HPO₄, 5 glucose, 10 lactate, 1 pyruvate, 20 HEPES, and 1% bovine serum albumin, pH 7.45, with the addition of 250 μCi/ml [³²P]orthophosphate (NEN Life Science Products). OK cells (2.0–2.5 mg of protein/dish) were labeled in the same buffer (2.5 ml/dish) containing 100 μCi/ml [³²P]orthophosphate for 5 h at 37 °C. All incubations with different agonists were performed at room temperature. The incubation was terminated by removing the medium, addition of immunoprecipitation buffer (100 mM NaCl, 50 mM Tris-HCl, 2 mM EGTA, 30 mM NaF, 30 mM Na₂O₄P₂, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 4 μg/ml aprotonin, and 1% Triton X-100, pH 7.45) and placing the samples on ice. The cells were disrupted by gentle homogenization. Immunoprecipitation of the Na,K-ATPase α subunit was performed as described (11). Briefly, aliquots (200 μg of protein) were incubated overnight at 4 °C with 50 μl of rabbit polyclonal antibody (primary experiments demonstrated that this antibody does not immunoprecipitate the wild type α subunit) and the simultaneous addition of excess protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). Samples were analyzed by SDS-polyacrylamide gel electrophoresis using the Laemmli system (31). Proteins were transferred to polyvinylidene difluoro membranes (Immobilon-P, Millipore, Bedford, MA) and subjected to autoradiography. Phosphoantibodies were analyzed by phosphoimaging, and quantitation was performed as described (4).

**Quantitation of Na,K-ATPase a Subunit Phosphorylation State—**In order to estimate the stoichiometry of the α subunit phosphorylation we used the "back phosphorylation" technique (32). PCT cells were incubated in

**References**

1. A. V. Chibalin, A. I. Katz, and A. M. Bertorello, unpublished observations.
the presence or absence of 1 μM dopamine at 23 °C for 2.5 min. The incubation was terminated by addition of homogenization buffer, and immunoprecipitation of the α subunit was performed as described above. The immunoprecipitated α subunit (∼0.25 μg) was phosphorylated by purified protein kinase C (77 μl/50 μl, 30 min at 30 °C in the presence of [γ-32P]ATP (NE-N Life Science Products), 10 mM MgCl2, 0.4 μM CaCl2, 0.25 mM EGTA, 0.03 mg/ml phosphatidyserine, 0.1 mg/ml bovine serum albumin, 100 μM Na3ATP, 20 mM Tris-Cl, pH 7.5. Samples were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue. The radioactivity incorporated into the α subunit excised from the gel was counted (Cherenkov), and the number of moles of phosphate incorporated per mole of α subunit was calculated as described (13). The amount of immunoprecipitated α subunit was calculated by scanning densitometry using bovine serum albumin as standard.

Preparation of Endosomes—OK cells in suspension (1.5 mg of protein/ml) were incubated under different protocols at room temperature. Incubation was terminated by transferring the samples to ice and addition of cold homogenization buffer containing 250 mM sucrose and 3 mM imidazole, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 4 μg/ml aprotinin, pH 7.4. Cells were gently homogenized (15–20 strokes) to minimize damage of the endosomes, using a pellet pestle motor homogenizer, and the samples were subjected to a brief (5 min) centrifugation (4 °C, 30,000 × g). Endosomes were fractionated on a flotation gradient as described (4), using essentially the technique of Corvel et al. (33). These fractions were not cross-contaminants, i.e. Rab5 was located exclusively in EE, whereas mannose 6-phosphate receptor immunoreactivity was located in LE (4).

Preparation of Clathrin-coated Vesicles—Isolation of clathrin vesicles was performed as described (4, 34). After preincubation with or without 1 μM DA (3 min at 25 °C), OK cells were homogenized using a Potter homogenizer (three strokes; 30 s) in 1 mM EGTA, 0.5 mM MgCl2, 0.1 mM MES, and 0.2 mg/ml Na3ATP, titrated to pH 6.5 with NaOH. The homogenate was centrifuged at 85,000 × g for 1 h, and the pellet was resuspended in the same buffer and applied to a discontinuous sucrose gradient (v/v): 60, 50, 40, 10, and 5%. Samples were then centrifuged at 80,000 × g for 75 min and collected from the 10–40% interface; they were washed in homogenization buffer and pelleted at 85,000 × g for 1 h. Wheat germ agglutinin was added to a concentration of 1:10 mg of protein/ml and incubated overnight at 4 °C. The agglutinated material was sedimented at 20,000 × g for 15 min. CCV were negatively stained for both Rab5 and mannose 6-phosphate receptor (4).

Preparation of Basolateral Plasma Membranes—After separation of early and late endosomes, another fraction (500 μl) was collected at the 16 and 42% sucrose interface corresponding to cell ghosts, mitochondria, and plasma membranes. Basolateral plasma membranes were further purified as described (11).

Miscellaneous—Protein content was determined according to Bradford (35). Western blots were developed with an ECL or ECL Plus (Amersham, Amersham, UK) detection kit. Scans were performed using a Scan Jet IIc scanner (Hewlett-Packard, Palo Alto, CA). Quantitation of the phosphorylated Na+,K+-ATPase α subunit was performed as described (11) using a Fuji Bas 1000 Bio-imaging analyzer (Fuji Co., Tokyo, Japan), and the data (arbitrary units) were analyzed using a software Tina 2.07 ray test (Isotopenmessyerei GmbH, Staulenhardt, Germany).

Statistics—Comparison between two experimental groups were made by the nonpaired Student’s t test. For multiple comparisons one way analysis of variance with Sheffe’s correction was used. p < 0.05 was considered significant.

RESULTS

Effect of Subunit Endocytosis on Na+,K+-ATPase Activity—The Na+,K+-ATPase α subunit is phosphorylated in response to dopamine, maximal phosphorylation (∼40%) being achieved after 2.5 min incubation at 23 °C with 1 μM dopamine. Using back phosphorylation we have observed in two independent experiments that dopamine decreased the amount of dephospho-α subunit (dephosphoprotein, percent of control: 67 (experiment 1) and 70 (experiment 2), Fig. 1A). Because the total amount of immunoprecipitated Na+,K+-ATPase α subunit was the same, the results further demonstrated that the α subunit is phosphorylated in the presence of dopamine. The α subunit immunoprecipitated from vehicle-treated cells was phosphorylated to a stoichiometry of 0.33 (experiment 1)/0.38 (experiment 2) mol/mol, whereas that from dopamine-treated cells to 0.20 (experiment 1)/0.25 (experiment 2) mol/mol, respectively. This indicates that in intact cells dopamine phosphorylates the Na+,K+-ATPase α subunit with a stoichiometry of ∼0.15 mol/mol (∼40% of maximal).

To determine the relative roles of α subunit phosphorylation and internalization during inhibition of Na+,K+-ATPase activ-
activity in PCT cells, we examined the effect of dopamine on Na\(^+\),K\(^+\)-ATPase activity and \(\alpha_1\) subunit phosphorylation under conditions where endocytosis was blocked. We have reported previously that preincubation with wortmannin or LY294009 prevented the endocytosis of \(\alpha_1\) subunits into clathrin vesicles and early and late endosomes induced by dopamine (10). In the present study we assessed the effect of 1 \(\mu\)M dopamine on Na\(^+\),K\(^+\)-ATPase activity and \(\alpha_1\) subunit phosphorylation in PCT cells that have been treated with 100 \(\mu\)M wortmannin (Wt) or 25 \(\mu\)M LY 294009 (LY) for 20 min at 23 °C before incubation with the ligand (Fig. 1). Dopamine alone increased the state of phosphorylation of the immunoprecipitated \(\alpha_1\) subunit from the basolateral membrane (Fig. 1A) and decreased Na\(^+\),K\(^+\)-ATPase activity (Fig. 1B). Dopamine-induced phosphorylation was not altered by wortmannin or LY294009 (Fig. 1A). However, the inhibition of Na\(^+\),K\(^+\)-ATPase activity was blocked by pretreatment with either wortmannin or LY294009 (Fig. 1B). Wortmannin or LY294009 alone did not affect Na\(^+\),K\(^+\)-ATPase activity or \(\alpha_1\) subunit phosphorylation. These results suggest that phosphorylation is a necessary, but not a sufficient, event for Na\(^+\),K\(^+\)-ATPase inhibition, i.e. although it triggers the endocytosis, it is the removal of the subunits from the plasma membrane, rather than the increased phosphorylation per se that decreases Na\(^+\),K\(^+\)-ATPase activity in intact cells.

Effect of S11A and S18A Mutations on Na\(^+\),K\(^+\)-ATPase Activity—To determine the role of putative PKC phosphorylation residues during inhibition of Na\(^+\),K\(^+\)-ATPase activity by dopamine, OK cells were transfected with mutants of the rat \(\alpha_1\) subunit in which either Ser-11 or Ser-18 were substituted by Ala residues. OK cells expressing wild type (Wt) and the mutants S11A and S18A \(\alpha_1\) subunit expressed comparable amounts of \(\alpha_1\) subunits as determined by Western blot analysis (Fig. 2A), and the Na\(^+\),K\(^+\)-ATPase activity determined in plasma membranes was also similar (Fig. 2B). In intact OK cells dopamine (1 \(\mu\)M; 3 min at 23 °C) decreased significantly \((p < 0.01, n = 4)\) Na\(^+\),K\(^+\)-ATPase activity (Fig. 2C), and this inhibition was abolished by coincubation with 1 \(\mu\)M Bis. In OK cells expressing the S11A isoform the inhibitory effect of 1 \(\mu\)M dopamine was slightly less than that in cells expressing the Wt isoform (Wt; ~40% versus S11A: ~30%), but it was still significant \((p < 0.01, n = 6)\). This effect as well was abolished by 1 \(\mu\)M Bis. In contrast, dopamine failed to induce a significant change in Na\(^+\),K\(^+\)-ATPase activity in cells expressing the S18A mutant (Fig. 2C).

It has been reported recently (36) that COS-7 cells expressing the Na\(^+\),K\(^+\)-ATPase \(\alpha_1\) isoform carrying a mutation in a protein kinase C phosphorylation residue (S23A, corresponding to Ser-18 in our study) fail to adhere properly to fibronectin or plastic matrix. We have therefore examined whether OK cells seeded on plastic coverslips at a density of 350 (Wt), 250 (S11A) and 260 (S18A) cells/\(\mu\)l and grown in 2.5 ml Dulbecco's modified Eagle's medium exhibit any culture
abnormalities. A representative photomicrograph demonstrates that after 16 h of culture, all OK cell types (Wt, S11A, and S18A) adhere and establish cell-to-cell contacts (Fig. 2D) and reach confluence within 36 h. Although we have not determined the intracellular sodium concentration or pH of the different OK cells examined, they did not display any differences in cell shape or size, e.g. an enlargement that would indicate a change in cell volume due to an abnormal intracellular ionic composition. Thus, one can speculate that the difference between these two studies could reflect merely the different cell lines used (COS-7 versus OK) rather than lack of the residue phosphorylated by protein kinase C.

**Na\(^{+},K^{+}\)-ATPase \(\alpha_1\) Subunit Phosphorylation—**To examine the state of \(\alpha_1\) subunit phosphorylation OK cells were metabolically labeled with \[^{32}\text{P}\]orthophosphate and then incubated with or without 1 \(\mu\text{M}\) dopamine (3 min; 23 °C). The degree of phosphorylation of the \(\alpha_1\) subunit was determined by autoradiography (Fig. 3A) and corrected for the amount of immunoprecipitated material assessed by Western blot (Fig. 3A, lower panel, and B). Dopamine increased significantly \((p < 0.01, n = 6)\) the \(\alpha_1\) subunit phosphorylation in Wt cells as well as in OK cells expressing the S11A mutant. The increase in phosphorylation was similar in Wt and S11A cells, and both effects were blocked by Bis. Notably, dopamine failed to phosphorylate the \(\alpha_1\) subunit in OK cells expressing the S18A mutant.

**Endocytosis of Na\(^{+},K^{+}\)-ATPase \(\alpha_1\) Subunit—**As reported earlier (Fig. 1 of Ref. 4) inhibition of Na\(^{+},K^{+}\)-ATPase activity is the consequence of a redistribution of enzyme units between the plasma membrane and intracellular compartments. When we reexamined our data in quantitative fashion from three experiments (Fig. 4), it is shown that upon incubation with dopamine a similar proportion (\(-40\%)\) of \(\alpha_1\) subunits that leave the plasma membrane-enriched fraction \((B)\) appear in the endosome-enriched fraction \((C)\), whereas the proportion of \(\alpha_1\) subunits in fraction A (lysosomes, mitochondria, endoplasmic reticulum) does not change significantly.

While the analysis of such distribution using cell fractionation on sucrose gradients is useful to calculate the proportional shift of subunits between these two compartments, it does not allow the accurate identification of the specific organelles into which the subunits are incorporated. Thus, to better illustrate the destination of the endocytosed \(\alpha_1\) subunits, and the differences in distribution when mutants are used, we examined whether phosphorylation of Ser-18 is essential for endocytosis by assessing the abundance of \(\alpha_1\) subunits in early and late endosomes. OK cells (Wt, S11A, and S18A) in suspension were incubated with or without 1 \(\mu\text{M}\) dopamine at 23 °C (Fig. 5) for 10 min (although different from that used to measure phosphorylation (3 min), this incubation time was chosen to enable us to detect an increase of \(\alpha_1\) subunit abundance in both early and late endosomes (see Ref. 4)). Dopamine increased the abundance of \(\alpha_1\) subunits in early and late endosomes in Wt \((p < 0.05, n = 4)\) and to a somewhat lesser extent in early endosomes from S11A \((p < 0.05, n = 4)\); this effect was blocked in both groups by coinubcation with the PKC inhibitor Bis. Bis alone had no significant effect on \(\alpha_1\) subunit abundance (not shown). However, in OK cells expressing the Na\(^{+},K^{+}\)-ATPase \(\alpha_1\) subunit carrying the S18A mutation, dopamine failed to induce a significant change in \(\alpha_1\) subunit abundance in either early or late endosomes (Fig. 5, lower panel). This observation adds further support for a causal relationship between phosphorylation of Ser-18 and endocytosis of the \(\alpha\) subunit in response to dopamine.

**Na\(^{+},K^{+}\)-ATPase \(\alpha_2\) Subunit Abundance in Clathrin-coated Vesicles—**To determine whether phosphorylation of Ser-18 was also required for clathrin-dependent internalization of the \(\alpha_1\) subunit, CCVs were prepared from OK cells expressing the Wt or S18A \(\alpha_2\) subunit (Fig. 6). The S11A mutant was not studied in this protocol, because this mutation does not affect the ability of dopamine to phosphorylate the \(\alpha\) subunit nor to inhibit its activity. OK cells in suspension were incubated (2.5 min) with or without 1 \(\mu\text{M}\) DA at 23 °C. While dopamine treatment increased \(\alpha_2\) subunit abundance in CCV from Wt cells \((p < 0.05, n = 4)\), it failed to do so in OK cells carrying the S18A mutation.
DISCUSSION

The results of this study demonstrate that dopamine-dependent inhibition of \(\text{Na}^+,\text{K}^+\)-ATPase activity in intact cells is caused by a reduced number of active units within the plasma membrane. Phosphorylation of the \(\text{Na}^+,\text{K}^+\)-ATPase \(\alpha\) subunit in the basolateral plasma membrane occurs at the Ser-18 residue, but this event per se does not modify the enzyme's catalytic activity. Rather, the decreased \(\text{Na}^+,\text{K}^+\)-ATPase activity in intact cells is caused by the removal of pump molecules from the basolateral plasma membrane and internalization by endocytosis, for which phosphorylation of the catalytic subunit appears to serve as a triggering signal.

Dopamine increased the state of \(\text{Na}^+,\text{K}^+\)-ATPase \(\alpha\) subunit phosphorylation by approximately 40% (Fig. 1B). In addition, back phosphorylation of the immunoprecipitated \(\alpha\) subunit demonstrated a reduced amount of dephosphoprotein of 30% (Fig. 1A). PKC phosphorylated the immunoprecipitated \(\alpha\) subunit from vehicle treated cells to a stoichiometry of 0.35 mol/mol and of 0.20 mol/mol from dopamine-treated cells. Thus, we have estimated that dopamine phosphorylates the \(\text{Na}^+,\text{K}^+\)-ATPase to a stoichiometry of 0.15 mol of phosphate/mol of \(\alpha\) subunit, which constitutes 40% of maximal stoichiometry (0.35 mol/mol). The stoichiometry of \(\alpha\) subunit phosphorylation has been reported to range between 0.33 and 1.0 mol/mol for the rat \(\alpha\) subunit, whereas in species lacking Ser-18 it has been shown to be no higher than 0.15 mol/mol (Ref. 18 and references therein). While those studies were performed using purified preparations (\(\text{Na}^+,\text{K}^+\)-ATPase and protein kinase C), the stoichiometry achieved in intact cells in response to a physiologic agonist has not been reported before.

We have shown previously that activation of GPCRs by dopamine inhibits \(\text{Na}^+,\text{K}^+\)-ATPase activity in renal epithelial cells and that this inhibition was associated with endocytosis of its \(\alpha\) and \(\beta\) subunits (4). Internalization occurs via clathrin vesicle formation and sequential transport into early and late endosomes. This process requires stimulation of phosphatidyl-
inositol 3-kinase activity, the time course of which coincides with the increased appearance of α subunits in the CCV compartment. The increased abundance of α subunits in CCV, EE, and LE was blocked by two phosphatidylinositol 3-kinase inhibitors, wortmannin or LY294009 (10), which differ in their potency, selectivity, and mode of action. The use of these compounds provides therefore a convenient model for testing whether preventing endocytosis affects the degree of α subunit phosphorylation in the plasma membrane and/or the changes in its catalytic activity in response to dopamine. In this study the magnitude of phosphorylation in the presence of dopamine was similar to that reported previously (11) and was not affected by the presence of either wortmannin or LY294009. However, the ability of dopamine to inhibit Na⁺,K⁺-ATPase activity was blunted if PCT cells were pretreated with either of the two phosphatidylinositol 3-kinase inhibitors (Fig. 1). The inhibitors by themselves did not significantly affect the degree of phosphorylation or of constitutive endocytosis, suggesting that during nonstimulated conditions these two processes have a relatively long half-life.

Failure of dopamine to inhibit Na⁺,K⁺-ATPase activity when endocytosis was prevented is in agreement with previous observations. Using a different approach, we have reported that stabilizing the cortical actin cytoskeleton inhibited the traffic of Na⁺,K⁺-ATPase subunits into CCV, EE, and LE and that under these conditions dopamine failed to inhibit Na⁺,K⁺-ATPase activity (4). Using the microtubule depolymerizing agent nocodazole, α subunit incorporation was impaired only at the endosomal level, and under this condition dopamine efficiently inhibited Na⁺,K⁺-ATPase activity, suggesting that subunit internalization in CCVs is sufficient to cause a decrease in Na⁺,K⁺-ATPase activity. In aggregate, these observations strongly suggest that it is the removal of the α subunit from the plasma membrane rather than its phosphorylation that results in decreased cell Na⁺,K⁺-ATPase activity, a mechanism perhaps analogous to that of other ion transport proteins such as the epithelial sodium channel (1) and H⁺/K⁺-ATPase (2).

Using site-directed mutagenesis we also demonstrated in this study that endocytosis of Na⁺,K⁺-ATPase and inhibition of its catalytic activity in response to DA require phosphorylation of the Ser-18 residue located in the α₁ subunit NH₂ terminus. Mutation of Ser-11, another α₁ subunit residue phosphorylated in vitro by PKC (16), did not affect the ability of DA to increase phosphorylation and stimulate internalization of the α₁ subunit nor the inhibitory action of DA on the enzymatic activity.

The Ser-11 and Ser-18 residues of the Na⁺,K⁺-ATPase α₁ subunit that undergo phosphorylation have been identified either in cell-free preparations by utilizing purified PKC (16) or in intact cells after activation of PKC by phorbol esters (23, 37). The former study established that approximately 75% of the phosphorylated subunit corresponded to Ser-18, whereas the rest was localized to Ser-11 (16), a distribution whose physiological relevance is uncertain. Our study suggests that in response to a physiological agonist (dopamine) only Ser-18 in the rat α₁ subunit is phosphorylated and that this is essential for endocytosis of the α subunit and inhibition of Na⁺,K⁺-ATPase activity. Substitution of Ser-11, although a PKC substrate (in cell-free preparations of Na⁺,K⁺-ATPase), did not affect its activity elicited by dopamine, suggesting that the Ser-11 does not represent a regulatory site in this process in the rat. It is possible, however, as proposed by Vasilets (23), that in other species (lacking Ser-18) Ser-11 represents the PKC phosphorylation site that modulates Na⁺,K⁺-ATPase activity and perhaps α₁ subunits endocytosis.

In summary, Na⁺,K⁺-ATPase activity in intact cells repre-
sents the number of active units within the basolateral plasma membrane. Decreased Na\(^+\),K\(^+\)-ATPase activity in response to GPCRs signals (dopamine) in renal epithelial cells is determined by removal of the subunits from the plasma membrane (Fig. 7). Phosphorylation occurs at Ser-18 of the α subunit and triggers this process, but it does not in itself affect the intrinsic properties of the enzyme. Once the molecules are internalized they become inactive, as the increased abundance of α and β subunits in intracellular compartments is not accompanied by a parallel increase in Na\(^+\),K\(^+\)-ATPase catalytic activity (4). Finally, some of the subunits may become dephosphorylated in late endosomes (11). This may be a step that serves as a signal for their recruitment to the plasma membrane (existence of such a regulatory pathway has been recently described in lung epithelial cells (38)) and/or their function in these organelles may be necessary for controlling membrane potential and intracellular pH, important factors required for vesicle sorting (39, 40).

REFERENCES

1. Shimkets, R. A., Lifton, R. P., and Canessa, C. M. (1997) J. Biol. Chem. 272, 25537–25541
2. Courtois-Coutry, N. D., Roush, D., Rajendran, V., McCarthy, J. B., Geibel, J., Kashgarian, M., and Caplan, M. (1997) Cell 90, 501–510
3. Schmalzing, G., Eckard, P., Kroener, S., and Passow, H. (1990) Am. J. Physiol. 258, C179–C184
4. Chibalin, A. V., Katz, A. I., Berggren, P.-O., and Bertorello, A. M. (1997) J. Biol. Chem. 272, C1458–C1465
5. Bertorello, A. M., and Katz, A. I. (1993) Am. J. Physiol. 265, F743–F755
6. Pierce, B. M., and Robinson, M. S. (1990) Annu. Rev. Cell Biol. 4, 573–586
7. Gruenberg, J., and Maxfield, F. R. (1995) Curr. Opin. Cell Biol. 7, 552–563
8. Kirchhausen, T., Bonifacino, J. S., and Reizman, H. (1997) Curr. Opin. Cell Biol. 9, 488–495
9. Chibalin, A. V., Katz, A. I., Berggren, P.-O., and Bertorello, A. M. (1998) Mol. Biol. Cell 9, 1299–1220
10. Chibalin, A. V., Pedemonte, C. H., Katz, A. I., Feraillle, E., Berggren, P.-O., and Bertorello, A. M. (1998) J. Biol. Chem. 273, 8814–8819
11. Lowndes, J. M., Hokin-Neaversen, M., and Bertics, P. J. (1990) Biochim. Biophys. Acta 1052, 143–151
12. Bertorello, A. M., Aperia, A., Walaas, I., Nairn, A. C., and Greengard, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11359–11362
13. Chibalin, A. V., Vasilets, L. A., Hennekes, H., Pralong, D., and Geering, K. (1992) J. Biol. Chem. 267, 22378–22384
14. Nishi, A., Bertorello, A. M., and Aperia, A. (1993) Acta Physiol. Scand. 149, 377–384
15. Feschenko, M. S., and Sweadner, K. J. (1995) J. Biol. Chem. 270, 14072–14077
16. Feschenko, M. S., and Sweadner, K. J. (1994) J. Biol. Chem. 269, 30436–30444
17. Feschenko, M. S., and Sweadner, K. J. (1997) J. Biol. Chem. 272, 17726–17733
18. Bertorello, A. M., and Aperia, A. (1998) Am. J. Physiol. 236, F370–F373
19. Ominato, M., Sato, T., and Katz, A. I. (1996) J. Membr. Biol. 152, 235–243
20. Bertorello, A. M. (1992) J. Cell Sci. 101, 343–347
21. Middleton, J. P., Khan, W. A., Collingsworth, G., Hannun, Y. A., and Medford, R. M. (1993) J. Biol. Chem. 268, 15958–15962
22. Vasilets, L. A. (1997) Cell. Physiol. Biochem. 7, 1–18
23. Feraillle, E., Carranza, M.-L., Buffin-Meyer, B., Rousselot, M., Doucet, A., and Favre, H. (1995) Am. J. Physiol. 268, C1277–C1283
24. Carranza, M.-L., Feraillle, E., and Favre, H. (1996) Am. J. Physiol. 271, C136–C143
25. Pedemonte, C. H., Pressley, T. A., Cinelli, A. R., and Lokhandwala, M. F. (1997) Mol. Pharmacol. 52, 88–97
26. Berson, J., Foster, I., Beguio, P., Geering, K., and Verrey, P. (1997) Mol. Biol. Cell 8, 387–398
27. Shull, G. E., Greh, J., and Lingrel, J. B. (1986) Biochemistry 25, 8125–8132
28. Birukov, K. G., and Bonifacino, J. S. (1996) J. Biol. Chem. 271, 22421–22427
29. Lamaze, C., and Schmid, S. L. (1995) Curr. Opin. Cell Biol. 7, 573–586
30. Erusalimsky, J. D., Friedberg, I., and Rozengurt, E. (1988) J. Biol. Chem. 263, 19188–19194
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Nestler, E., and Greengard, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 77, 7479–7483
33. Gorvel, J.-P., Chavrier, P., Zerial, M., and Gruenberg, J. (1995) Cell 81, 915–925
34. Hammond, T. G., and Verroust, P. G. (1994) Am. J. Physiol. 266, F554–F562
35. Bradford, M. M. (1974) Anal. Biochem. 268, 248–254
36. Belusa, R., Wang, Z.-M., Matsubara, T., Sahlgren, B., Kulubova, I., Nairn, A. C., Russelait, E., Greengard, P., and Aperia, A. (1997) J. Biol. Chem. 272, 20179–20184
37. Logvinenko, N. S., Kulubova, I., Fedosova, N., Larsson, S. H., Nairn, A. C., Easman, M., Greengard, P., and Aperia, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9132–9137
38. Bertorello, A. M., Ridge, K., Chibalin, A. V., Katz, A. I., and Sznajder, J. I. (1999) Am. J. Physiol., in press
39. Fuchs, R., Schmid, S., and Mellman, I. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5229–5234
40. Cain, C. C., Sipe, D. M., and Murphy, R. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 544–548

Na\(^+\),K\(^+\)-ATPase Phosphorylation and Endocytosis 1927

...tracellular pH, important factors required for vesicle sorting...
Dopamine-induced Endocytosis of Na\textsuperscript{+},K\textsuperscript{+}-ATPase Is Initiated by Phosphorylation of Ser-18 in the Rat α Subunit and Is Responsible for the Decreased Activity in Epithelial Cells

Alexander V. Chibalin, Goichi Ogimoto, Carlos H. Pedemonte, Thomas A. Pressley, Adrian I. Katz, Eric Féraille, Per-Olof Berggren and Alejandro M. Bertorello

J. Biol. Chem. 1999, 274:1920-1927.
doi: 10.1074/jbc.274.4.1920

Access the most updated version of this article at http://www.jbc.org/content/274/4/1920

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 18 of which can be accessed free at http://www.jbc.org/content/274/4/1920.full.html#ref-list-1