J. Membrane Biol. 191, 25–36 (2002)
DOI: 10.1007/s00232-002-1043-3

The Journal of
Membrane
Biology

Trafficking of Na,K-ATPase Fused to Enhanced Green Fluorescent Protein Is Mediated by Protein Kinase A or C

B. Kristensen[1], **S. Birkelund**[2], **P.L. Jorgensen**[1]

[1]Biomembrane Center, The August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen OE, Denmark

[2]Department of Medical Microbiology and Immunology, University of Aarhus, Wilh Meyers Allé, Building 240, DK-8000 Aarhus C, Denmark

Received: 29 April 2002/Revised: 14 August 2002

Abstract. Fusion of enhanced green fluorescent protein (EGFP) to the C-terminal of rat Na,K-ATPase α1-subunit is introduced as a novel procedure for visualizing trafficking of Na,K-pumps in living COS-1 renal cells in response to PKA or PKC stimulation. Stable, functional expression of the fluorescent chimera (Na,K-EGFP) was achieved in COS-1 cells using combined puromycin and ouabain selection procedures. Na,K-pump activities were unchanged after fusion with EGFP, both in basal and regulated states. In confocal laser scanning and fluorescence microscopes, the Na,K-EGFP chimera was distributed mainly along the plasma membrane of COS cells. In unstimulated COS cells, Na,K-EGFP was also present in lysosomes and in vesicles en route from the endoplasmic reticulum to the plasma membrane, but it was almost absent from recycling endosomes labelled with fluorescent transferrin. After activation of protein kinase A or C, the density of co-localizing Na,K-EGFP and transferrin vesicles was increased 3–4-fold, while the ouabain-sensitive ^{86}Rb uptake was reduced by 22%. Simultaneous activation of PKA and PKC had additive effects with a 6-fold increase of co-localization and a 38% reduction of ^{86}Rb uptake. Responses of similar magnitude were seen after inhibition of protein phosphatase by okadaic acid. Reduction of the amount of Na,K-ATPase in surface plasma membranes through internalization in recycling endosomes may thus in part explain a decrease in Na,K-pump activity following protein kinase activation or protein phosphatase inhibition.

Key words: Na,K-ATPase — EGFP-α1 subunit chimera — Recycling endosomes — Lysosomes — Protein kinase A and C

Introduction

The Na,K-pump maintains Na^+ and K^+ gradients that provide the driving force for secondary active transport processes of other solutes in kidney and intestine, for propagation of electrical signals, and for regulation of cell volume. Regulation of the active Na, K-transport in kidney tubules and other epithelial cells bordering the extracellular phase is also important for blood pressure control [16]. Knowledge of the molecular basis for modulation of Na,K-ATPase activity and for the expression and transport of the Na,K-pump protein in cells is important for understanding these essential processes.

The rat renal Na,K-pump consists of the α1-subunit with 1018 residues and β1-subunit with 304 residues and three N-linked glycosylations [29]. The α1-subunit can be phosphorylated by protein kinases, PKA and PKC in vitro [5, 18] and in intact cells [17, 18, 19, 37]. Phosphorylation of serine or threonine in transfected cells is important for the short-term regulation of Na,K-ATPase activity or pump rate, but the consequences of the modification differ, depending on the assay conditions. Some authors reported activation [14, 37], while several groups provided evidence for inactivation [3, 6, 9, 18, 35] of Na,K-ATPase after stimulation of PKC. Isoforms of PKC are also of importance, since only the classical PKCs

Abbreviations: EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; IBMX, 3-isobutyl-1-methylxanthine; IRES, internal ribosome entry site; Na,K-EGFP, EGFP fused to the carboxy terminal of rat α1 Na,K-ATPase subunit; OA, ocadaic acid; PKA, cAMP dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

Correspondence to: P.L. Jorgensen; email: pljorgensen@aki.ku.dk

(α, βI, γ) phosphorylate the α subunit of Na,K-ATPase and inhibit enzyme activity [26]. Short-term reduction of activity after phosphorylation may involve changes in poise of the E_1-E_2 equilibrium [31] and altered affinity for Na^+ [16] or it can be due to internalization of pump molecules into endosomes [9, 10, 35]. In skeletal muscle, recruitment of α2-Na,K-ATPase from intracellular vesicles to the plasma membrane is seen in response to insulin [24, 32] or exercise [25]. In proximal tubule cells, both PKC- and PKA-mediated internalization of α1-Na,K-ATPase in clathrin-coated vesicles and endosomes has been identified by sucrose gradient centrifugation, flotation gradients, and SDS-PAGE analysis [9]. In OK cells, immuno-fluorescence studies revealed co-localization of clathrin and Na,K-pump protein following PKC activation via dopamine treatment [35]. In A6 epithelial cells, PMA (phorbol 12-myristate 13-acetate) stimulation of PKC reduced the Na,K-ATPase activity through internalization, but this reduction in basolateral surface area was independent of the phosphorylation state of Na,K-ATPase [4]. Increased Na,K-ATPase activity due to recruitment of enzyme protein from endosomal stores to the plasma membrane was observed in alveolar cells after β-adrenergic stimulation or PKA activation, but the effect did not depend on the phosphorylation state of the PKA site in Na,K-ATPase [7]. In COS-7 cells, PKA activation significantly inhibited the intrinsic Na,K-ATPase activity without altering ouabain binding to the cell surface [1]. The inhibition may therefore not be due to internalization of the enzyme from the plasma membrane.

Direct, real-time visualization of the trafficking of Na,K-ATPase molecules between the surface membrane and intracellular vesicles is a valuable tool for determining whether a distinct internalization mechanism is involved in changes of Na,K-ATPase activity following activation of protein kinase A or C. Coexpression of protein fused with enhanced green fluorescent protein has been developed as a reliable and inert probe for visualization of important cellular processes [20, 34]. Here, a fusion protein consisting of rat α1 subunit of Na,K-ATPase and enhanced green fluorescent protein was stably expressed as fully functional, fluorescent Na,K-pumps in COS-1 cells using a combination of puromycin- and ouabain-selection procedures [27]. This ensures homogeneous fluorescence labeling of the Na,K-EGFP cell population, thus avoiding the heterogeneity often observed after transient expression of EGFP fusion proteins. The distribution of the chimera in surface membranes and intracellular vesicles could then be examined in confocal laser-scanning and fluorescence microscopes. To quantify internalization, red transferrin was used as a label of the recycling endosomal pool and the density of internalized green vesicles containing Na,K-EGFP was estimated by counting a large number of fluorescence images of COS-1 cells following activation of protein kinase A or C or inhibition of protein phosphatase.

Materials and Methods

Cell Culture

COS-1 cells were maintained at 37°C in an atmosphere of 5% CO_2 and a humidity of 100%. Cells were grown in Dulbecco's modified Eagle Medium supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Cell culture reagents were purchased through GIBCO Life Technologies, Denmark.

Construction of Plasmid pMC-EGFP

A vector pMC-EGFP, containing enhanced green fluorescent protein (EGFP) under transcriptional control of a CMV (cyto megalo virus) promoter was constructed and kindly donated by Christian Mielke, University of Würzburg, Germany. The plasmid pMC-2P was used for the construction of bicistronic expression vectors for mammalian cells [33]. EGFP is expressed together with the puromycin resistance gene from a single bicistronic transcript. The EGFP gene is inserted in front of the IRES element, an internal ribosome entry site from poliovirus [39] that mediates the translational initiation of the selection marker representing the second cistron. For insertion of EGFP in front of the IRES, the open reading frame of EGFP was amplified by PCR from pEGFP-1 (CLONTECH Laboratories, Germany). PCR primers added restriction sites for *Hind*III, *Not*I, *Mlu*I, *Bsu*36I, *Spe*I, and *Apa*I to the 5′ end and an *Eco*RI site to the 3′ end, and the fragment was cloned into the *Hind*III and *Eco*RI sites of pMC-2P yielding plasmid pMC-EGFP.

Construction of Plasmid pRat-α1

Full-length rat α1 Na,K-ATPase cDNA was kindly donated by Jerry Lingrel, University of Cincinatti, USA, and used as template for PCR amplification of rat α1. Primers were designed to introduce *Sal*I sites up- and downstream of rat α1. The PCR fragment was inserted as a *Sal*I- rat α1-*Sal*I fragment in the *Xho*I site of pcDNAI/Neo (Invitrogen Corporation, UK) resulting in plasmid pRat-α1. Correct orientation of rat α1 was verified by sequencing.

Construction of Plasmid pNa,K-EGFP

The pRat-α1 plasmid was used as template for PCR amplification of rat α1. Primers were designed to introduce a *Not*I site upstream of α1 and an *Apa*I site downstream. The PCR fragment was inserted in pMC-EGFP as a *Not*I-rat α1-*Apa*I fragment yielding plasmid pNa,K-EGFP. The nucleotide composition between rat α1 and EGFP introduced a linker region of 7 amino acid residues (gly-gly-pro-pro-val-ala-thr) in the fusion protein. To obtain puromycin resistance, transcription of the bicistronic messenger also encoding Na,K-EGFP is obligatory.

Transfection of COS-1 Cells

Transfection of COS-1 cells with pNa,K-EGFP was performed with FuGENE™6 reagent (Roche Biochemicals, Denmark) as previously described [45] at a DNA/lipid ratio of 0.35 μg/μl. Selection and expansion of positive transfectants was performed

with 2 μg/ml puromycin for 3 weeks [46]. To select for Na,K-EGFP functionality, cells resistant to puromycin were further treated with 10 μM ouabain for 3 weeks [27]. Ouabain-resistant colonies were re-cloned, expanded into stable clones, and subsequently maintained in 10 μM ouabain. Transfection with pRat-α1 was carried out in a similar fashion, except for the omission of a puromycin selection step.

Membrane Preparation

COS-1 cells were trypsinated of dishes, pelleted (1.000 × *g*, 4°C, 5 min) and resuspended in 25 mM imidazole-HCl, 1 mM EDTA, 1 mM EGTA, 10% sucrose, pH 7,5. Membranes were subsequently prepared as previously described [38].

SDS Gels and Western Blotting

Electrophoresis and western blotting were performed as previously described [38]. The primary polyclonal antibody (rabbit) was raised against the large intracellular loop of Na,K-ATPase α1 subunit (pig). This antibody cross-reacts with α isoforms from multiple species, including rat Na,K-ATPase. The secondary antibody (pig) was a polyclonal alkaline phosphatase conjugated anti-rabbit antibody (DAKO, Denmark).

^{86}Rb-Uptake Assays

COS-1 cells were trypsinized and resuspended to 500,000 cells/ml at 37°C; 30 min prior to the assay, 100 nM phorbol-12-myristate-13-acetate (PMA) or 100 μM forskolin + 1 mM 3-isobutyl-1-methylxanthine (IBMX) was added to activate PKC or PKA, or 100 nM ocadaic acid (OA) to inhibit protein phosphatase PP-1 and PP-2A. Medium contained either 10 μM ouabain to inhibit the COS-1 endogenous Na,K-pump or 2 mM ouabain to inhibit the expressed rat α1 wild type or rat α1 Na,K-EGFP pumps. Bumetanide, 50 μM, was added to inhibit the $Na^+K^+2Cl^-$ cotransporter, since active K^+-transport through this system may also be regulated by protein kinases [23]. ^{86}RbCl was added to the growth medium (0.5 μCi/ml) and after 0, 10, and 20 min, aliquots of 1 ml were transferred to Eppendorf tubes containing 200 μl of silicon oil and cells and media were separated by an oil centrifugation technique [28]. The oil, which had a higher density than the suspending medium but lighter than the suspended cells, consisted of 77% w/w AR 200 oil (SERVA, Boehringer Ingelheim Bioproducts Partnership, Germany) and 23% w/w 200 DC 20 oil (Bie & Berntsen, Denmark). Cell pellets were resuspended in 1200 μl of water and aliquots of 1000 μl were counted in a gamma counter. Rubidium uptake through wild-type rat α1 Na,K-pump or Na,K-EGFP-pump were determined as the counts from cells in medium with 10 μM ouabain minus those in 2 mM ouabain medium. Native COS-1 ^{86}Rb uptake was likewise determined from counts in medium devoid of ouabain minus those in medium with 10 μM, ouabain. The $^{86}Rb^+$ fraction of total K^+ in the growth medium was taken into account when calculating potassium uptake.

Fluorescence Microscopy

The day before microscopy, 50,000 cells were plated on chambered coverglass (NUNC plasticware, Denmark) in 2 ml CO_2-independent medium (GIBCO Life Technologies, Denmark) enriched with 10% fetal bovine serum and 2 mM glutamine. The medium was changed 2–4 hours prior to fluorescence microscopy. Microscopy was done at 37°C with an inverted Leitz DMRBE fluorescence microscope (Leica Microskopie und Systeme, Wetzlar, Germany).

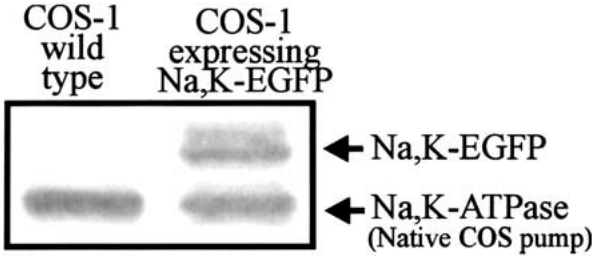

Fig. 1. Western blot of membrane proteins. 5 μg of total membrane protein was separated on 8% SDS gels. The epitope for the primary antibody was located in the large intracellular loop of Na, K-ATPase. An alkaline phosphatase-conjugated secondary antibody was used. Membranes were scanned using a phosphoimager.

Images were collected through a video camera (Sony 3CCD) connected to a HP workstation.

Loading of Cells with Fluorescent Transferrin

Cells plated on chambered coverglass were loaded with 100 μg/ml Texas Red-labelled ferro-transferrin [21], (Molecular Probes, Oregon, U.S.A.) in CO_2 independent medium for 2 hours. The last 45 minutes of the loading protocol was performed with or without stimulation of PKA (100 μM forskolin + 1 mM IBMX) or PKC (100 nM PMA) and inhibition of protein phosphatases (100 nM okadaic acid). Subsequently, cells were washed twice with medium and subjected to fluorescence microscopy (excitation, 595 nm; emission, 615 nm).

Loading of Cells with Fluorescent Dextran

Cells plated on chambered coverglass were grown in medium (CO_2-independent) with 200 μg/ml cascade blue dextran [36] (Molecular Probes, Oregon, U.S.A.) for 24 hours, washed twice with medium, maintained in fresh medium for 2 hours, washed in medium, and subjected to fluorescence microscopy (excitation, 400 nm; emission, 420 nm).

Confocal Laser Scanning Microscopy (CLSM)

Cells were plated according to the fluorescence microscopy procedure described above. CLSM was performed with a Leica TCS confocal laser-scanning microscope using an OS-9 computer for image collection. The numerical aperture of the objective lens (PL APO 100 from Leica) was 1.4 and structures in sections with a thickness of 0.6 μm were discernible. After defining top and bottom of cells, excitation was performed at 450–490 nm with a suppression filter of 520 nm. Images of 12 sections were collected. Each section was scanned 8 times and electronically averaged to reduce background noise. Vertical step size was approximately 1.2 μm.

Results

Expression of Na,K-EGFP in COS-1 Cells

The average abundance of rat α1-subunit fused in the chimera, Na,K-EGFP, in five membrane preparations was 90 ± 13% of that of the native α1-subunit in COS-1 cells, as estimated by densitometry of western blots of membrane preparations (Fig. 1).

Table 1. Effects of forskolin, PMA and ocadaic acid on ^{86}Rb uptake

	^{86}Rb uptake (nmol $K^+/10^6$ cells per min)		
	Native COS-1	rat α1 wild type	Na,K-EGFP
Control	4.9 ± 0.4	5.5 ± 0.3	4.7 ± 0.5
F + I	3.8 ± 0.5[a]	4.0 ± 0.4[c]	3.7 ± 0.1[b]
PMA	5.1 ± 0.3	4.3 ± 0.1[b]	3.6 ± 0.3[b]
F + I & PMA	4.0 ± 0.3[a,e]	3.2 ± 0.3[c,d,f]	2.9 ± 0.1[c,d,e]
OA	4.1 ± 0.2[a,e]	4.4 ± 0.3[a]	3.6 ± 0.3[b]

Data are estimated by linear regression analysis of ^{86}Rb uptake after incubation of the cells at 37°C for 0, 10 and 20 min. Results are average values of three experiments ± SD. F + I: 100 μM forskolin and 1 mM IBMX, PMA: 100 nM PMA, OA: 100 nM ocadaic acid. Assays were performed as described in Materials and Methods. [a]indicates $P < 0.05$, [b]indicates $P < 0.01$, and [c]indicates $P < 0.001$ compared to control values, [d]indicates $P < 0.05$ compared to PKA activation, [e]indicates $P < 0.05$ and [f]indicates $P < 0.01$ compared to PKC activation. ANOVA was performed on the data and the Student-Newman-Keuls multiple comparisons test was used to estimate the significance of differences between mean values.

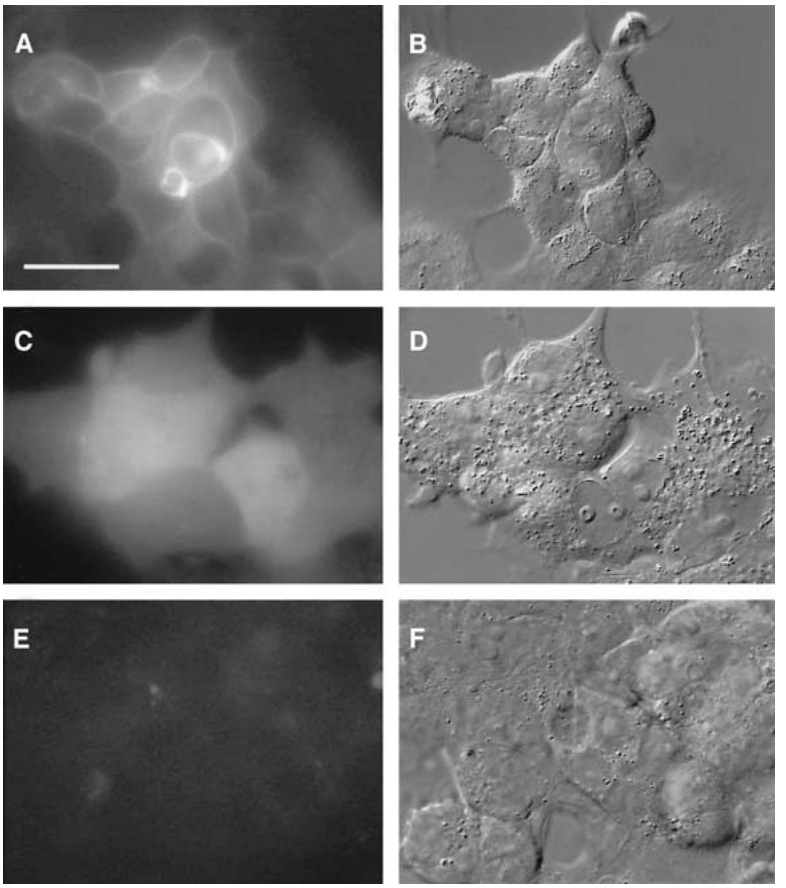

Fig. 2. Low-magnification fluorescence images of COS-1 cell cultures. (*A*, *C* and *E*) Fluorescence pictures (excitation, 488 nm; emission, 519 nm).(*B*, *D* and *F*) Normaski pictures. (*A*, *B*) COS-1 cells expressing Na,K-EGFP. (*C*, *D*) COS-1 cells expressing EGFP alone. (*E*, *F*) Wild-type COS-1 cells. Na,K-EGFP was distinctly localized along the periphery of cells (*A*), while EGFP alone was diffusely distributed in cells (*C*). Untreated COS cells showed no fluorescence (*E*). Scale bar = 20 μm.

Although COS-1 cells expressing ouabain-resistant Na,K-EGFP had been maintained in 10 μM ouabain for 25 generations, the abundance of intrinsic, ouabain-sensitive COS-1 Na,K-ATPase remained at 98 ± 5% of that in untransfected COS-1 cells (Fig. 1).

Cation Transport Properties of Na,K-EGFP in COS-1 Cells

The rate of the ouabain-sensitive component of ^{86}Rb uptake in COS-1 cells expressing Na,K-EGFP was 86% of the rate in cells expressing wild-type rat α1 and 96% of the rate in native COS-1 cells (Table 1). These rates correspond well to the relative amounts of α-subunit, as estimated above from western blots (Fig. 1). The fusion with EGFP did therefore not suppress the turnover rate of the Na,K-pump in the conditions of the transport assay.

Activation of PKA or PKC suppressed the Na,K-pump activity after expression of either the rat α1-subunit alone or the rat α1-subunit in a chimera with EGFP. Data in Table 1 show that the average

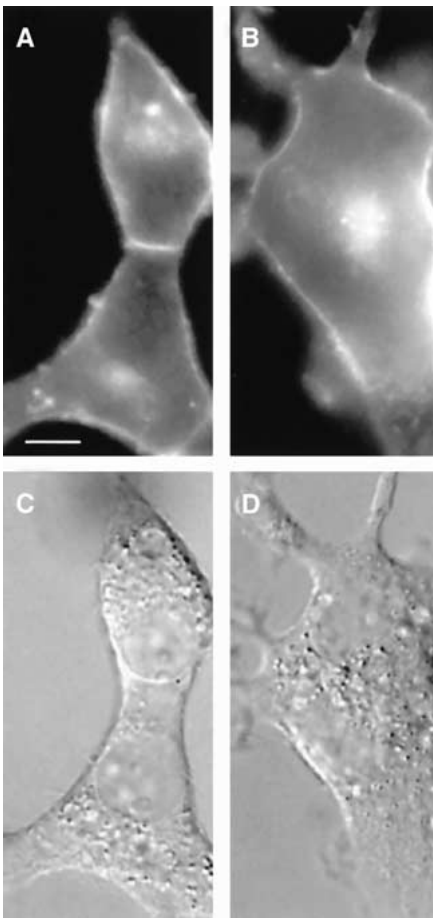

Fig. 3. Typical COS-1 cells expressing Na,K-EGFP. (*A*, *B*) Fluorescence pictures of Na,K-EGFP (excitation, 488 nm; emission, 519 nm). (*C*, *D*) Normaski pictures. Na,K-EGFP was present at the cell periphery, in a juxtanuclear compartment and in distinct spots in the cytoplasm. Scale bar = 5 μm.

reduction of $^{86}Rb^{+}$ uptake after activation of PKA was 27% in COS-1 cells expressing the rat α1-subunit and 21% in COS-1 cells expressing Na, K-EGFP. The corresponding figures for PKC were 22% and 23%, respectively. Simultaneous activation of PKA and PKC had almost additive effects with reductions of 42% and 38% of the rates of $^{86}Rb^{+}$ uptake in COS-1 cells expressing rat α1-subunit and Na,K-EGFP, respectively. In the presence of 100 nM ocadaic acid, the inhibitor of protein phosphatases 1 and 2A, the uptake of ^{86}Rb was reduced to levels similar to those observed after activation of PKA or PKC, Table 1. Fusion of EGFP to the C-terminus of rat α1-subunit Na,K-ATPase therefore neither altered the basal Na, K-transport activity nor the regulatory effects of PKA or PKC on the rat α1 Na,K-pump in COS-1 cells. In contrast to the rat α1-Na,K-pump, the native COS-1 Na,K-pump was not inhibited by PKC activation, while PKA or ocadaic acid reduced ^{86}Rb uptake by 22% or 16%, respectively.

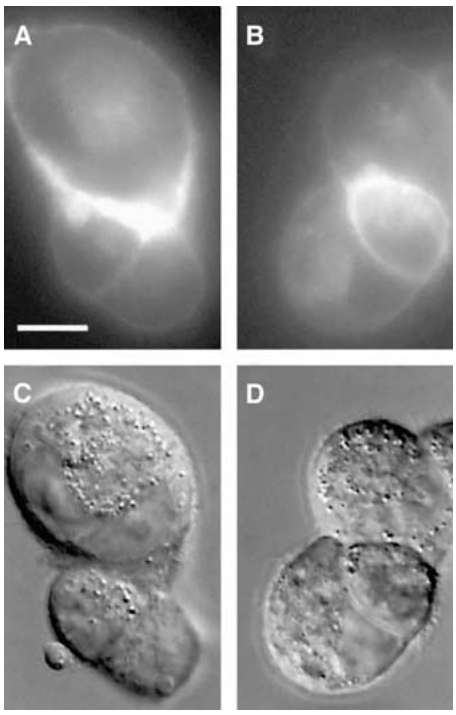

Fig. 4. Polarization of COS-1 cells. (*A*, *B*) Fluorescence pictures of Na,K-EGFP in aggregated COS cells with parts of the plasma membrane in close proximity. (*C*, *D*) Normaski pictures. Levels of fluorescence were much higher at the junctional area than anywhere else along the cell periphery. Scale bar = 10 μm.

For the experiments in Table 1, cells were incubated in medium containing 50 μM bumetanide. In the absence of bumetanide, the PKC-mediated reduction in rate of ouabain-sensitive $^{86}Rb^{+}$ uptake in COS cells expressing Na,K-EGFP was larger (35 ± 4%) than in the presence of bumetanide (23%) (*data not shown*). It is known that PKC may activate the Na,K,2Cl cotransporter activity [23] and this should be taken into account when comparing the data in Table 1 with previous results [8]. In unstimulated wild-type COS-1 cells the relative contributions to $^{86}Rb^{+}$ uptake were 60% from the Na,K-pump, 25% from the Na,K,2Cl cotransporter, and 15% from other pathways (*data not shown*).

Visualization of Na,K-EGFP in COS-1 Cells

The fluorescence of the chimera, Na,K-EGFP (Fig. 2*A*), was distinctly localized along the periphery of cells. In contrast, fluorescence was diffusely distributed throughout the cytoplasm of COS-1 cells expressing EGFP alone, without Na,K-ATPase α1 subunit (Fig. 2*C*). Untransfected wild-type COS-1 cells did not exhibit fluorescence (Fig. 2*E*).

Intracellular details are discernible in the images of COS-1 cells expressing Na,K-EGFP in Fig. 3. In addition to the distinct lining along the cell periphery corresponding to the plasma membrane, an intense

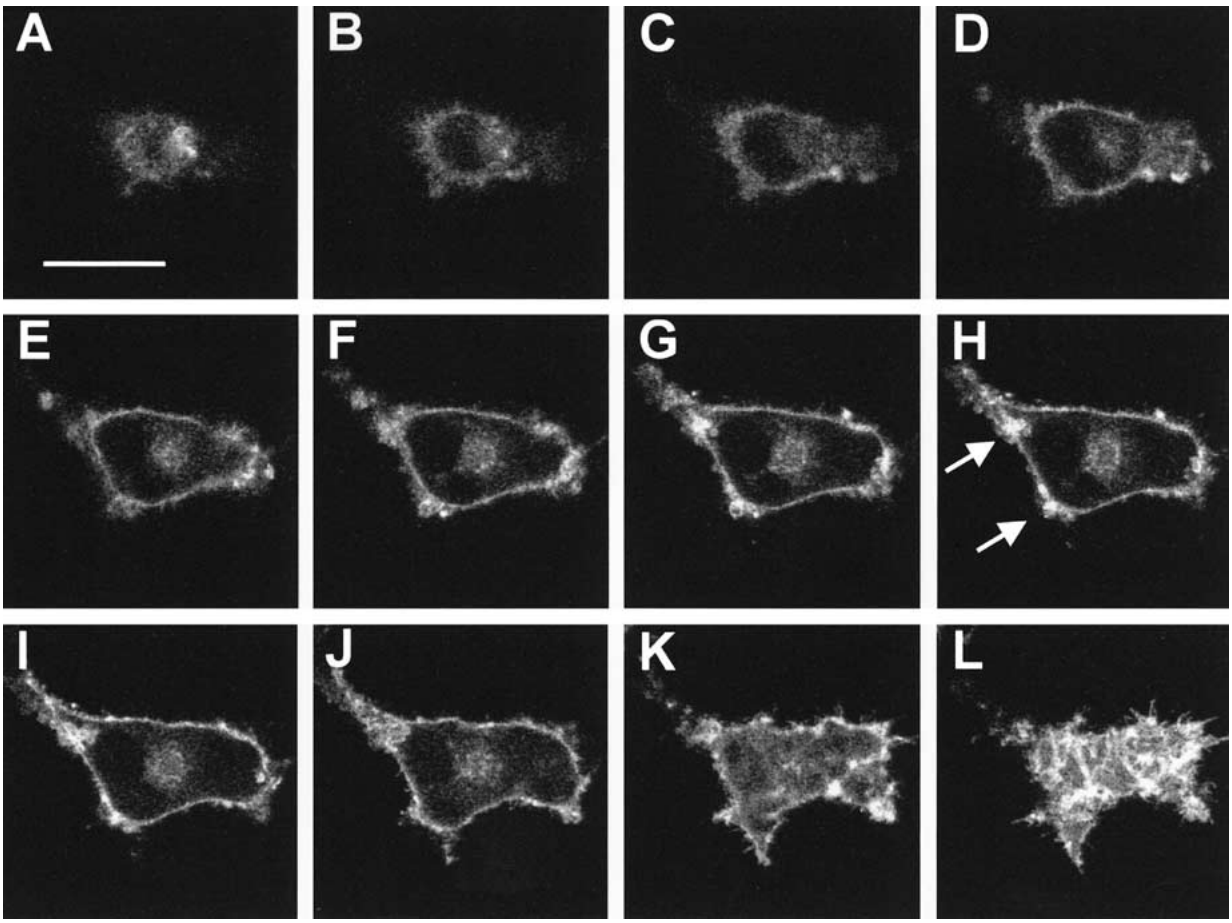

Fig. 5. Confocal laser scanning microscopy of a COS-1 cell expressing Na,K-EGFP. Images were taken in 1.2-μm vertical steps from (*A*) top of cell to (*L*) plane of attachment. Na,K-EGFP was present everywhere along the periphery of cells as well as in a distinct intracellular pool. Arrows (in *H*) indicate regions with particularly high fluorescence. Scale bar = 20 μm.

fluorescence of Na,K-EGFP was also visible near the nuclei, presumably corresponding to the endoplasmic reticulum (ER) and Golgi complex. Fluorescent spots in the cytoplasm of cells correspond to vesicles containing Na,K-EGFP. Another interesting aspect is the distribution of Na,K-pumps in areas of the cells in close proximity to their neighbors. The images of untreated COS-1 cells in Fig. 4 show that Na,K-EGFP was present at much higher local concentrations at the junction area between cells, as compared to the plasma membrane directly facing the extracellular growth medium. COS cells in culture may therefore retain the ability to accumulate Na, K-ATPase in plasma membrane areas engaging in cell-cell contact. This resembles the distribution of Na,K-ATPase along basolateral infoldings in kidney tubular cells [40].

Confocal laser scanning microscopy was performed to further characterize the distribution of Na,K-EGFP in COS-1 cells. Fig. 5 shows that fluorescence of Na,K-EGFP is seen as a continuous lining along the plasma membrane, including the membrane at the plane of attachment with the cover glass (Fig. 5*L*). The intensity of fluorescence at the plane of attachment resembles that in areas of cell-cell contact described above (Fig. 4). Certain areas of the plasma membrane had higher local concentrations of Na,K-EGFP than their surroundings (arrows, Fig. 5*H*). Apart from the plasma membrane, Na,K-EGFP was again visible at a juxtanuclear intracellular store, probably the ER/Golgi complex (clearly visible in Fig. 5*E–J*).

Distribution of Na,K-EGFP on Cytoplasmic Vesicle Compartments

Markers were introduced to localize Na,K-EGFP α1-subunit to defined vesicle compartments in the cytoplasm of COS-1 cells: fluorescent dextran (cascade blue, MW 10,000) as a lysosomal marker [36] and fluorescent transferrin (Texas Red) as a marker of recycling endosomes [21]. For labelling of lysosomes, cells were incubated with fluorescent dextran overnight. Subsequent growth of cells in dextran-free media for 2 hours emptied the endosomal compartment of dextran to leave the labelling exclusively in

Table 2. Effects of forskolin, PMA and ocadaic acid on the density of vesicles containing Texas Red transferrin, Na,K-EGFP or both

	Density (vesicles per 100 μm^2 section)		
	Transferrin	Na,K-EGFP	Colocalization
Control	2.7 ± 0.4	1.0 ± 0.2	0.09 ± 0.03
F + I	2.5 ± 0.3	1.7 ± 0.2	0.6 ± 0.2[b]
PMA	2.3 ± 0.2	1.2 ± 0.2	0.4 ± 0.1[a]
F + I & PMA	2.3 ± 0.2	2.2 ± 0.2[b,f]	1.1 ± 0.1[c,d,g]
OA	1.9 ± 0.3	1.6 ± 0.1	0.8 ± 0.1[c,f]
OA, F + I & PMA	2.3 ± 0.2	2.1 ± 0.3[b,e]	1.0 ± 0.2[c,d,g]

For quantification, square grids (5 × 5 μm) were superimposed on fluorescence images of Texas Red transferrin-loaded COS-1 cells expressing Na,K-EGFP. The number of green (Na,K-EGFP) or red (Texas Red-labeled transferrin) vesicles were counted and the density was estimated. Colocalization denotes vesicles containing both Na,K-EGFP and transferrin. F + I: 100 μM forskolin and 1 mM IBMX; PMA: 100 nM PMA; OA: 100 nM ocadaic acid. All results are average values ± SEM ($n > 12$). [a]indicates $P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$ as compared to control values, [d]indicates $P < 0.01$ as compared to F + I values. [e]indicates $P < 0.05$, [f]$P < 0.01$, and [g]$P < 0.001$ as compared to PMA values. ANOVA was performed on the data and the Student-Newman-Keuls multiple comparisons test was used to estimate the significance of differences between mean values.

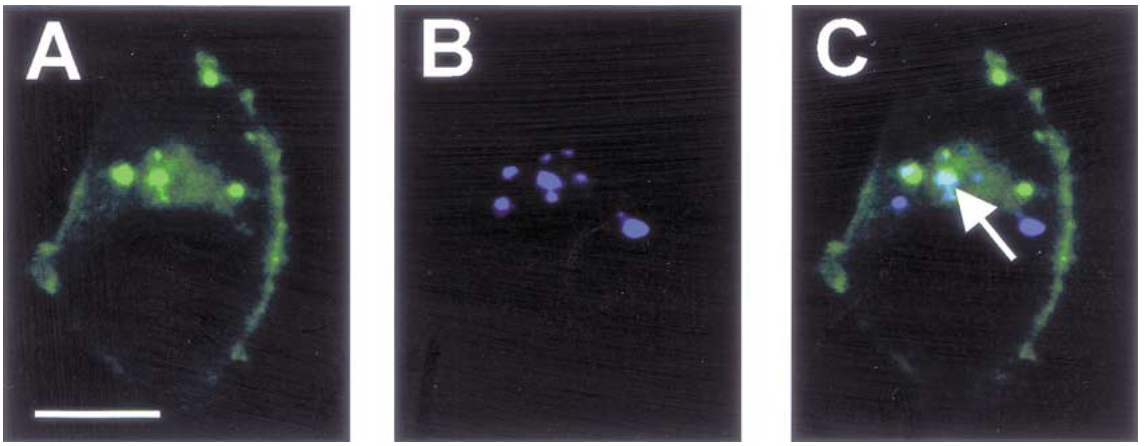

Fig. 6. Unstimulated COS-1 cell loaded with Cascade Blue dextran (200 μg/ml). (*A*) Na,K-EGFP (excitation, 488 nm; emission, 519 nm). (*B*) Cascade Blue dextran (excitation, 400 nm; emission, 420 nm). (*C*) Overlays (Adobe Photoshop 6.0) of *A* and *B*. Several vesicles contain both Na,K-EGFP and Cascade Blue dextran, indicating the presence of Na,K-pump in lysosomes. The arrow in Fig. 6*C* points to such a vesicle. Scale bar = 10 μm.

the lysosomal compartment. After counting a number of images ($n = 11$; method described in legend to Table 2), the average density of vesicles containing Cascade Blue dextran was estimated to be 1.7 ± 0.2 per 100 μm^2 of cell section area. The density of Na,K-EGFP vesicles was 1.2 ± 0.2 and that of overlying blue and green vesicles (*see* Fig. 6) was 0.6 ± 0.1 per 100 μm^2. Hence, in nonstimulated COS cells, about 50% of cytoplasmic Na,K-EGFP vesicles were lysosomal in nature. A similar estimation of the density of endosomes in Texas-Red transferrin-loaded COS cells showed that 9% of Na,K-EGFP vesicles in control cells were recycling endosomes (Table 2). The remaining pool of Na,K-EGFP vesicles (41%) could be involved in the transport of newly synthesized Na,K-ATPase from the Golgi apparatus to the plasma membrane. One example is shown in Fig. 7, where two distinct vesicles are traced en route to fusion with the plasma membrane.

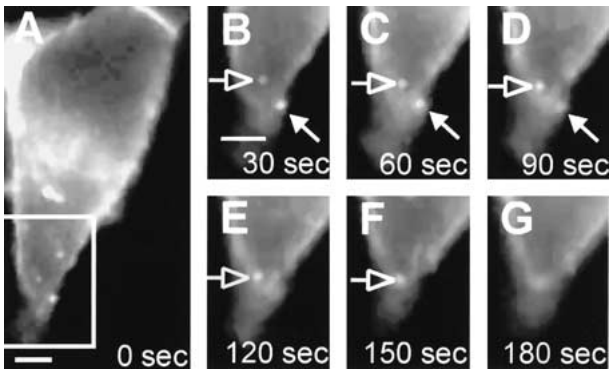

Fig. 7. A temporal study of a COS-1 cell expressing Na,K-EGFP. (*A*) Fluorescence picture of Na,K-EGFP. (*B–G*) Magnification of the area defined by white tracing in (*A*). Pictures were taken at 30-sec intervals at 37°C. Two vesicles (indicated by different styles of arrows) are seen fusing with the plasma membrane. Scale bar = 2 μm.

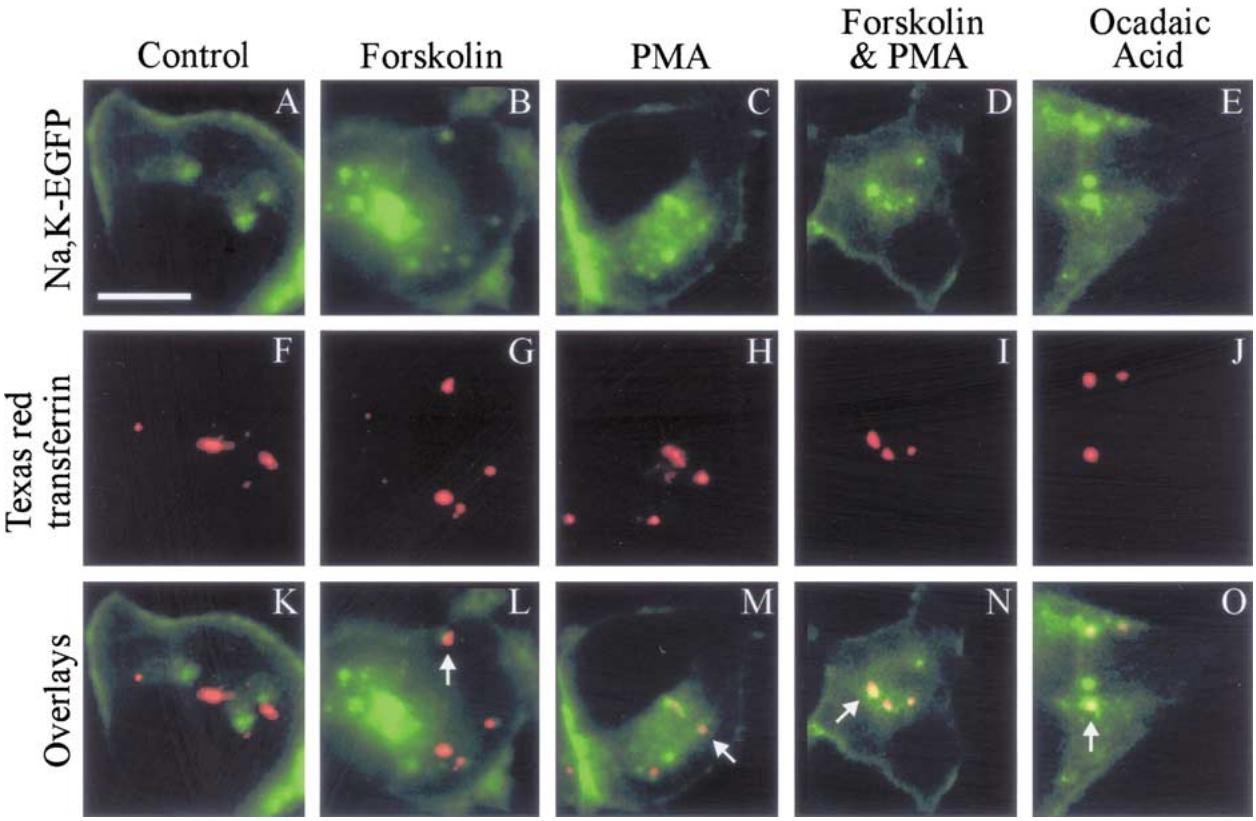

Fig. 8. COS-1 cells loaded with Texas Red transferrin (100 μg/ml). (*A–E*) Na,K-EGFP (excitation, 488 nm; emission 519 nm). (*F–J*) Texas Red transferrin (excitation, 595 nm; emission, 615 nm). (*K–O*): Overlays (Adobe Photoshop 6.0) of *A* and *F*, *B* and *G*, *C* and *H*, *D* and *I*, and *E* and *J*, respectively. In untreated cells (*A*, *F*, *K*), there was no colocalization of Na,K-EGFP and Texas Red transferrin (*K*). After activation of PKA (*B*, *G*, *L*) or PKC (*C*, *H*, *M*), colocalization was observed (*L*, *M*). Simultaneous PKA and PKC activation (*D*, *I*, *N*) led to a high degree of colocalization (*N*). Inhibition of PP-1 and PP-2A (*E*, *J*, *O*) also led to colocalization (*O*). Arrows in Fig. 8 *L–O* indicate vesicles containing both Na,K-EGFP and Texas Red transferrin. PKA was activated with 100 μM forskolin and 1 mM IBMX, PKC was activated with 100 nM PMA, and PP-1 and PP-2A were inhibited with 100 nM okadaic acid. Scale bar = 10 μm.

The Influence of Protein Phosphorylation on the Trafficking of Na,K-EGFP-Labelled Vesicles

After loading with Texas Red transferrin, COS cells expressing Na,K-EGFP were treated with forskolin and IBMX to activate PKA, with PMA to activate PKC, or with ocadaic acid to inhibit protein phosphatases. Fluorescence images of Na,K-EGFP vesicles, Texas Red transferrin-labelled vesicles, and overlays of the two sets of images are shown in Fig. 8 and the estimated vesicle densities are given in Table 2. In control cells, colocalization of vesicles labelled with Na,K-EGFP and red transferrin was limited to 9% of Na,K-EGFP-labelled vesicles in the cytoplasm. After stimulation of PKA (Fig. 8*B*, *G*, *L*) or PKC (Fig. 8*C*, *H*, *M*), the density of overlying vesicles containing both Na,K-EGFP and red transferrin increased significantly, about 4-fold to 35% or 33% of the total density of Na,K-EGFP vesicles, respectively (Table 2). Simultaneous activation of PKA and PKC (Fig. 8*D*, *I*, *N*) increased the fraction of overlying vesicles about 6-fold, to 50% of the total density of Na,K-EGFP vesicles (Table 2). Similarly, after ocadaic-acid inhibition of protein phosphatases (Fig. 8*E*, *J*, *O*), the fraction of colocalized vesicles was increased to 48% of the total density of Na,K-EGFP vesicles (Table 2).

The total density of Na,K-EGFP-containing vesicles was also increased up to 2-fold, but this effect was statistically significant only for simultaneous PKA and PKC activation with or without concomitant inhibition of protein phosphatases (Table 2). In these experiments, the density of Na,K-EGFP vesicles was increased to 2.1–2.2 vesicles per 100 μm^2 as compared with 1.0 Na,K-EGFP vesicles per 100 μm^2 in control cells. In parallel, the fraction of Na,K-EGFP vesicles overlying vesicles labelled with red transferrin was increased from 0.09 to 1.0–1.1 (Table 2), suggesting that most of the increment in density is due to transfer of Na,K-EGFP to the compartment of recycling endosomes.

The average radius of vesicles containing both Na,K-EGFP and red transferrin was 0.4 μm, corre-

sponding to vesicle areas of 2.0 μm^2 ($4\pi r^2$). In spite of the large increment in the density of endosomal Na,K-EGFP vesicles following PKA and PKC activation (Table 2), the total membrane area of internalized vesicles forms only about 2% of the surface membrane area. This level of internalization can only explain the 21–42% inhibition of ^{86}Rb-uptake after activation of PKA and PKC (Table 1) if the density of Na,K-pumps is much higher in endosomal vesicle membranes than in the surface membrane. Electronmicroscopic observations, indeed, show that receptor densities can be increased 5–15-fold by internalization [42]. In basolateral membranes of kidney cells, the density of Na,K-ATPase is often high, up to 5000 per μm^2, and approaching a maximum value. Large increments of Na,K-pump densities in the membranes of endosomal vesicles, therefore, appear less probable than for the more disperse receptor units. In addition to internalization, activation of PKA and PKC may therefore evoke other mechanisms [16, 31] for reducing the activity of Na,K-pumps in the cell surface membrane.

Discussion

Functional expression of rat α1 Na,K-EGFP can be achieved in COS-1 cells using a combination of puromycin- [46] and ouabain-selection procedures [27]. This approach allows direct visualization of the redistribution of Na,K-pumps in response to external stimuli and thus represents a new approach at studies of the distribution of Na,K-ATPase in living mammalian cell lines. The quantitative assays of chimeric protein expression and active K^+ uptake in the COS-1 cells show that both the basal and the PKA- or PKC-stimulated Na,K-pump activity was unaffected by the fusion with EGFP.

In basal conditions, the fluorescence of Na,K-EGFP shows the expected distribution along the surface membrane of the COS-1 cells and in cytoplasmic organelles (Fig. 3). Accumulation is also seen in plasma membrane areas engaging in cell-cell contact (Fig. 4) as well as in the plane of attachment (Fig. 5), suggesting that Na,K-ATPase in COS cell cultures have preserved the cytoskeletal associations that are responsible for the subcellular distribution of Na,K-ATPase along the basolateral membranes of epithelial cells of kidney nephrons [40].

The COS-1 cells expressing Na,K-EGFP can be maintained in 10 μM ouabain indefinitely (more than 25 generations), and the ouabain-resistant chimera maintains active Na^+ and K^+ transport to cover the demands in the COS-1 cells, without the evidence for toxicity seen in other systems [30]. Na,K-EGFP is therefore a reliable marker of the cellular distribution and specific localization of functional Na,K-pumps in living COS-1 cells.

After 25 generations in medium supplemented with 10 μM ouabain, the amount of endogenous α1 subunit in Na,K-EGFP-expressing COS-1 cells was still equal to the amount in wild-type COS-1 cells (Fig. 1). In Na,K-EGFP-expressing COS-1 cells, the rat α1-EGFP fusion protein was also expressed at this level, implying that the transfected cells effectively expressed two-fold as much α-subunit as wild-type cells. Since the stability and translocation to the plasma membrane of the α-subunit depends on interaction with the β-subunit [2], the regulation of the synthesis of the β-subunit in COS-1 cells appears to be independent of the α-subunit regulation.

In control cells, most vesicles containing Na,K-EGFP were either lysosomes, as envisioned with cascade-blue dextran (Fig. 6), or storage- and transport vesicles on their way to the plasma membrane (Fig. 7). Colocalization of Na,K-EGFP and Texas Red transferrin was very modest in control cells (Fig. 8*K* and Table 2), suggesting that under basal conditions, limited amounts of Na,K-ATPase are present in an endosomal fraction recruitable to the plasma membrane in COS cells.

Following PKA or PKC stimulation, the fraction of vesicles containing both Na,K-EGFP and labelled transferrin was increased (Fig. 8*L* and *M* and Table 2) and it was further increased by simultaneous PKA and PKC activation (Fig. 8*N* and Table 2). PKA and PKC stimulation thus evoke individual mechanisms in COS-1 cells for internalization of Na,K-EGFP in early endosomes. This is accompanied by a 21–42% reduction of active K^+(Rb^+)-ion uptake (Table 1). Inhibition of protein phosphatases PP-1 and PP-2A with ocadaic acid mimicked the effect of simultaneous forskolin and PMA treatment on colocalization of Na,K-EGFP and transferrin (Fig. 8*O* and Table 2). This supports a regulatory model dependent on kinase- and phosphatase-mediated changes in phosphorylation states as opposed to unspecific actions of forskolin or PMA. Combining double kinase activation with phosphatase inhibition did not further increase the level of Na,K-EGFP/transferrin colocalization compared to phosphatase inhibition alone (Table 2), suggesting that Na,K-ATPase α1 subunit is continuously being phosphorylated and dephosphorylated by kinases and phosphatases, respectively. Under basal conditions, activity of protein phosphatases outweighs that of kinases, but during activated states (in this report, forskolin or PMA treatment) the kinase activity plays a more dominant role compared to phosphatases.

The colocalization of Na,K-EGFP pumps and fluorescent transferrin in recycling endosomes is compatible with the demonstration of internalization of Na,K-ATPase following protein kinase stimulation in previous studies employing different methods. Internalization was demonstrated by colocalization of antibodies to clathrin and Na,K-ATPase [35],

vesicle fractionation by ultra-centrifugation [9, 10] or ouabain binding and membrane capacitance measurements [4]. In one study on COS-7 cells, ouabain binding to native Na,K-pumps at the cell surface was unaltered after PKA stimulated inhibition of K^+ (Rb^+) uptake [1]. This could be due to the different assays of internalization or to different mechanisms of regulation of the native Na,K-ATPase in COS-7 cells as compared to the transfected rat Na,K-ATPase in COS-1 cells. Alternative mechanisms for changing Na, K-pump activity are also available, as it has been shown that protein kinase stimulation can change the conformational equilibrium [31] to alter the V_{max} and the apparent affinity for Na^+ [16, 37].

Previous studies based on the expression of rat α1 Na,K-ATPase in COS cells also reported a decrease in Na,K-pump activity following PKA or PKC stimulation [8, 18], in agreement with membrane fractionation studies on PCT and OK cells [9, 10, 35]. In response to PKC stimulation by dopamine, about 40% of the Na,K-pumps from the plasma membranes were relocated to endosomal fractions in parallel to a decrease in pump activity [10, 35]. In our findings, the ouabain-sensitive uptake of ^{86}Rb in COS cells expressing Na,K-EGFP was also reduced by PKA and PKC (Table 1) to the same extent as ^{86}Rb uptake in COS cells expressing wild-type rat Na,K-ATPase α1 subunit (Table 1). In apparent contrast to this, a decrease in intracellular Na^+ has been reported following PKC activation with PMA in OK cells [37]. It appears that PKC activation by PMA leads to concomitant phosphorylation of Ser11 (the consensus PKC site in α1 from most species) and Ser18 (a PKC site unique to the rodent α1 isoform) of rat α1 in OK cells and that this may elicit recruitment of Na,K-ATPase to the plasma membrane [14]. On the other hand, stimulation by dopamine leads to phosphorylation only of Ser18 and this may cause endocytosis of Na,K-ATPase [9, 10, 35]. Although both OK and COS cells are mammalian kidney cell lines, it is possible that fundamental differences between the cell types can account for the different effects of PMA and dopamine on Na,K-pump activity and distribution.

Based on alignment of the Na,K-ATPase sequence with the known structure of the E_1-form of Ca-ATPase, 1EUL [44], it was recently proposed that the PKA site (Ser938) is sterically inaccessible to the kinase [43]. In our hands, PKA activation led to internalization of Na,K-EGFP (Fig. 8*L* and Table 2) as well as inhibition of rubidium uptake (Table 1). To further address the problem, a Ser938→Ala mutation of rat α1 was constructed and expressed in COS-1 cells. This abolished the inhibitory effect of PKA activation on rubidium uptake (control: 5.3 ± 0.7 nmol $K^+/10^6$ cells per min; PKA activation: 5.3 ± 0.6 nmol $K^+/10^6$ cells per min, $n = 3$, using methods described in the legend to Table 1 — *data not shown*). It is therefore probable that the observed effects of PKA on Na,K-ATPase in the present study are caused by phosphorylation of the αl subunit. Examination of the three-dimensional structure of an E_2-form of Ca-ATPase (1FQU in the Protein Data Bank) also show altered steric relationships around the cytoplasmic loop between transmembrane segments 8 and 9, suggesting that serine-938 of Na,K-ATPase could be more accessible in the E_2-form than in the E_1-form. Another possibility is that phosphorylation at this conserved site close to the membrane depends on tethering of PKA to the membrane phase by targeting proteins [15].

In rat skeletal muscle, translocation of α2-Na,K-ATPase from an intracellular compartment to the plasma membrane in response to insulin [24, 32] or exercise [25] has been shown by membrane fractioning studies [24, 25] and immunoelectron microscopy [32]. The stimulatory effect of insulin on Na,K-ATPase α2 is dependent on phosphatidylinositol 3-kinase (PI 3 kinase) together with downstream protein phosphatase activation [12].

Interestingly, dopamine-mediated inhibition of the renal α1 isoform of Na,K-ATPase also depends on PI 3 kinase, but in conjunction with increased activity of serine kinases and internalization of Na, K-ATPase from the plasma membrane [9]. Protein kinase-mediated phosphorylation thus seems to cause endocytosis of Na,K-pumps from the plasma membrane, while protein phosphatase-mediated dephosphorylation causes exocytosis. Depending on tissue type and Na,K-ATPase subtype, the basal level of phosphorylation differs. In kidney cells, the α1 subtype is probably modestly phosphorylated, but activation of kinases increases the phosphorylation level and internalizes pump molecules. In muscle cells, an intracellular pool of presumably phosphorylated α2 Na,K-ATPase is present and can be recruited to the plasma membrane in response to protein phosphatase activation [12] following insulin treatment [12, 24, 32] or exercise [25].

It is important to consider the possibility that fusion of EGFP to the C-terminal of the α-subunit of Na,K-ATPase can alter the behavior of the pump protein with respect to cellular trafficking. In the present study, N-terminal fusion was avoided to preserve the access of PKC to the phosphorylation sites at Ser11 and Ser18 of the N-terminus. A recent paper, however, has shown that fusion of GFP with the N-terminal of rat α1 Na,K-ATPase does not interfere with dopamine-mediated endocytosis or inhibition of this subunit in OK cells [13]. The C-terminal fusion was not expected to interfere with PKA phosphorylation of Ser938 in the cytoplasmic loop between transmembrane segments 8 and 9 since this position is 80 residues upstream from the C-terminus. In other membrane proteins, the position of a fusion with GFP has had significant effects on intracellular

trafficking. Comparison of C-terminal and N-terminal fusion of GFP with AQP2 showed that only the N-terminal fusion recycled normally in response to vasopressin or forskolin, suggesting that the C-terminal fusion may interfere with a targeting motif that directs the water channel to a regulated pathway of exo- or endocytosis in response to cAMP [22]. Both C- and N-terminal fusion of GFP with GLUT4 exhibited insulin-dependent translocation to the plasma membrane, but unlike the native GLUT4 protein, these fusion proteins were not re-internalized following insulin removal [41]. The sorting signal involved in dopamine-mediated endocytosis of Na,K-ATPase in OK cells is tyrosine 537 of the α1 subunit [13]. In gastric H,K-ATPase, a tyrosine-based signal is also important for endocytosis from the luminal membrane of the parietal cells, but this residue is located in the β-subunit [11].

Christian Mielke, Department of Clinical Chemistry, Medizinische Poliklinik, University of Würzburg, Klinikstraße 6–8, D-97070 Würzburg, Germany, constructed and kindly donated vector pMC-EGFP, containing enhanced green fluorescent protein (EGFP) under transcriptional control of a CMV promoter (submitted for publication). The work was supported by the Danish Natural Sciences Research Council and the Novo-Nordic and Carlsberg Foundations.

References

1. Andersson, R.M., Cheng, S.X., Aperia, A. 1998. Forskolin-induced down-regulation of Na,K-ATPase activity is not associated with internalization of the enzyme. *Acta Physiol. Scand.* **164:**39–46
2. Beguin, P., Hasler, U., Staub, O., Geering, K. 2000. Endoplasmic reticulum quality control of oligomeric membrane proteins: topogenic determinants involved in the degradation of the unassembled Na,K-ATPase alpha subunit and in its stabilization by beta subunit assembly. *Mol. Biol. Cell.* **11:**1657–1672
3. Belusa, R., Wang, Z.M., Matsubara, T., Sahlgren, B., Dulubova, I., Nairn, A.C., Ruoslahti, E., Greengard, P., Aperia, A. 1997. Mutation of the protein kinase C phosphorylation site on rat alpha1 Na,K-ATPase alters regulation of intracellular Na^+ and pH and influences cell shape and adhesiveness. *J. Biol. Chem.* **272:**20179–20184
4. Beron, J., Forster, I., Beguin, P., Geering, K., Verrey, F. 1997. Phorbol 12-myristate 13-acetate down-regulates Na,K-ATPase independent of its protein kinase C site: decrease in basolateral cell surface area. *Mol. Biol. Cell.* **8:**387–398
5. Bertorello, A.M., Aperia, A., Walaas, S.I., Nairn, A.C., Greengard, P. 1991. Phosphorylation of the catalytic subunit of Na,K-ATPase inhibits the activity of the enzyme. *Proc. Natl. Acad. Sci. USA* **88:**11359–11362
6. Bertorello, A.M., Katz, A.I. 1993. Short-term regulation of renal Na,K-ATPase activity: physiological relevance and cellular mechanisms. *Am. J. Physiol.* **265:**F743–F755
7. Bertorello, A.M., Ridge, K.M., Chibalin, A.V., Katz, A.I., Sznajder, J.I. 1999. Isoproterenol increases Na,K-ATPase activity by membrane insertion of alpha-subunits in lung alveolar cells. *Am. J. Physiol.* **276:**L20–L27
8. Cheng, X.J., Hoog, J.O., Nairn, A.C., Greengard, P., Aperia, A. 1997. Regulation of rat Na,K-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am. J. Physiol.* **273:**C1981–C1986
9. Chibalin, A.V., Zierath, J.R., Katz, A.I., Berggren, P.O., Bertorello, A.M. 1998. Phosphatidylinositol 3-kinase-mediated endocytosis of renal Na,K-ATPase alpha subunit in response to dopamine. *Mol. Biol. Cell.* **9:**1209–1220
10. Chibalin, A.V., Ogimoto, G., Pedemonte, C.H., Pressley, T.A., Katz, A.I., Feraille, E., Berggren, P.O., Bertorello, A.M. 1999. Dopamine-induced endocytosis of Na,K-ATPase is initiated by phosphorylation of Ser-18 in the rat alpha subunit and Is responsible for the decreased activity in epithelial cells. *J. Biol. Chem.* **274:**1920–1927
11. Courtois-Coutry, N., Roush, D., Rajendran, V., McCarthy, J.B., Geibel, J., Kashgarian, M., Caplan, M.J. 1997. A tyrosine-based signal targets H/K-ATPase to a regulated compartment and is required for the cessation of gastric acid secretion. *Cell* **90:**501–510
12. Deachapunya, C., Palmer-Densmore, M., O'Grady, S.M. 1999. Insulin stimulates transepithelial sodium transport by activation of a protein phosphatase that increases Na,K-ATPase activity in endometrial epithelial cells. *J. Gen. Physiol.* **114:**561–574
13. Doné, S.C., Leibiger, I.B., Efendiev, R., Katz, A.I., Leibiger, B., Berggren, P.O., Pedemonte, C.H., Bertorello, A.M. 2002. Tyrosine 537 within the Na,K-ATPase alpha-subunit is essential for AP-2 binding and clathrin-dependent endocytosis. *J. Biol. Chem.* **277:**17108–17111
14. Efendiev, R., Bertorello, A.M., Pressley, T.A., Rousselot, M., Feraille, E., Pedemonte, C.H. 2000. Simultaneous phosphorylation of Ser11 and Ser18 in the alpha-subunit promotes the recruitment of Na,K-ATPase molecules to the plasma membrane. *Biochemistry* **39:**9884–9892
15. Feliciello, A., Gottesman, M.E., Avvedimento, E.V. 2001. The biological functions of A-kinase anchor proteins. *J. Mol. Biol.* **27:**99–114
16. Feraille, E., Doucet, A. 2001. Sodium-potassium-adenosine-triphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol. Rev.* **81:**345–418
17. Feschenko, M.S., Sweadner, K.J. 1997. Phosphorylation of Na,K-ATPase by protein kinase C at Ser18 occurs in intact cells but does not result in direct inhibition of ATP hydrolysis. *J. Biol. Chem.* **272:**17726–17733
18. Fisone, G., Cheng, S.X., Nairn, A.C., Czernik, A.J., Hemmings Jr., H.C., Hoog, J.O., Bertorello, A.M., Kaiser, R., Bergman, T., Jornvall, H. 1994. Identification of the phosphorylation site for cAMP-dependent protein kinase on Na,K-ATPase and effects of site-directed mutagenesis. *J. Biol. Chem.* **269:**9368–9373
19. Fisone, G., Snyder, G.L, Fryckstedt, J., Caplan, M.J., Aperia, A., Greengard, P. 1995. Na,K-ATPase in the choroid plexus. Regulation by serotonin/protein kinase C pathway. *J. Biol. Chem.* **270:**2427–2430
20. Gerdes, H.H., Kaether, C. 1996. Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389:**44–47
21. Ghosh, R.N., Gelman, D.L., Maxfield, F.R. 1994. Quantification of low density lipoprotein and transferrin endocytic sorting in Hep2 cells using confocal microscopy. *J. Cell Sci.* **107:**2177–2189
22. Gustafson, C.E., Levine, S., Katsura, T., McLaughlin, M., Aleixo, M.D., Tamarappoo, B.K., Verkman, A.S., Brown, D. 1998. Vasopressin regulated trafficking of a green fluorescent protein-aquaporin 2 chimera in LLC-PK1 cells. *Histochem. Cell Biol.* **110:**377–386

23. Haas, M., Forbush 3rd, B. 2000. The Na-K-Cl cotransporter of secretory epithelia. *Annu. Rev. Physiol.* **62:**515–534
24. Hundal, H.S., Marette, A., Mitsumoto, Y., Ramlal, T., Blostein, R., Klip, A. 1992. Insulin induces translocation of the alpha 2 and beta 1 subunits of the Na,K-ATPase from intracellular compartments to the plasma membrane in mammalian skeletal muscle. *J. Biol. Chem.* **267:**5040–5043
25. Juel, C., Nielsen, J.J., Bangsbo, J. 2000. Exercise-induced translocation of Na,K pump subunits to the plasma membrane in human skeletal muscle. *Am. J. Physiol.* **278:**R1107–R1110
26. Kazanietz, M.G., Caloca, M.J., Aizman, O., Nowicki, S. 2001. Phosphorylation of the catalytic subunit of rat renal Na,K-ATPase by classical PKC isoforms. *Arch. Biochem. Biophys.* **388:**74–80
27. Kent, R.B., Emanuel, J.R., Ben Neriah, Y., Levenson, R., Housman, D.E. 1987. Ouabain resistance conferred by expression of the cDNA for a murine Na,K-ATPase alpha subunit. *Science* **237:**901–903
28. Lew, V.L., Brown, A.M. 1979. Detection and Measurement of Free Ca^{2+} in Cells. pp. 423–432 Elsevier: Amsterdam, North-Holland
29. Lingrel, J.B., Orlowski, J., Shull, M.M., Price, E.M. 1990. Molecular genetics of Na,K-ATPase. *Prog. Nucleic Acid Res. Mol. Biol.* **38:**37–89
30. Liu, H.S., Jan, M.S., Chou, C.K., Chen, P.H., Ke, N.J. 1999. Is green fluorescent protein toxic to the living cells? *Biochem. Biophys. Res. Commun.* **260:**712–717
31. Logvinenko, N.S., Dulubova, I., Fedosova, N., Larsson, S.H., Nairn, A.C., Esmann, M., Greengard, P., Aperia, A. 1996. Phosphorylation by protein kinase C of serine-23 of the alpha-1 subunit of rat Na,K-ATPase affects its conformational equilibrium. *Proc. Natl. Acad. Sci. USA* **93:**9132–9137
32. Marette, A., Krischer, J., Lavoie, L., Ackerley, C., Carpentier, J.L., Klip, A. 1993. Insulin increases the Na,K-ATPase alpha 2-subunit in the surface of rat skeletal muscle: morphological evidence. *Am. J. Physiol.* **265:**C1716–C1722
33. Mielke, C., Tummler, M., Schubeler, D., von Hoegen, I., Hauser, H. 2000. Stabilized, long-term expression of heterodimeric proteins from tricistronic mRNA. *Gene* **254:**1–8
34. Misteli, T., Spector, D.L. 1997. Applications of the green fluorescent protein in cell biology and biotechnology. *Nat. Biotechnol.* **15:**961–964
35. Ogimoto, G., Yudowski, G.A., Barker, C.J., Köhler, M., Katz, A.I., Feraille, E., Pedemonte, C.H., Berggren, P.O., Bertorello, A.M. 2000. G protein-coupled receptors regulate Na,K-ATPase activity and endocytosis by modulating the recruitment of adaptor protein 2 and clathrin. *Proc. Natl. Acad. Sci. USA* **97:**3242–3247
36. Pagnano, R.E., Watanabe, R., Wheatley, C., Chen, C-S. 1999. Use of N-[5-(5,7-dimethyl boron dipyrromethene difluoride-sphingomyelin to study membrane traffic along the endocytic pathway. *Chem. Phys. Lipids.* **102:**55–63
37. Pedemonte, C.H., Pressley, T.A., Cinelli, A.R., Lokhandwala, M.F. 1997. Stimulation of protein kinase C rapidly reduces intracellular Na^+ concentration via activation of the Na^+ pump in OK cells. *Mol. Pharmacol.* **52:**88–97
38. Pedersen, P.A., Rasmussen, J.H., Jorgensen, P.L. 1996. Expression in high yield of pig $\alpha 1\beta 1$ Na, K-ATPase and inactive mutants D369N and D807N in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271:**2514–2522
39. Pelletier, J., Sonenberg, N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334:**320–325
40. Piepenhagen, P.A., Peters, L.L., Lux, S.E., Nelson, W.J. 1995. Differential expression of Na,K-ATPase, ankyrin, fodrin, and E-cadherin along the kidney nephron. *Am. J. Physiol.* **269:**C1417–C1432
41. Powell, K.A., Campbell, L.C., Tavare, J.M., Leader, D.P., Wakefield, J.A., Gould, G.W. 1999. Trafficking of Glut4-green fluorescent protein chimaeras in 3T3-L1 adipocytes suggests distinct internalization mechanisms regulating cell surface glut4 levels. *Biochem. J.* **344:**535–543
42. Rosenfeld, M.E., Bowen-Pope, D.F., Ross, R. 1984. Platelet-derived growth factor: morphologic and biochemical studies of binding, internalization, and degradation. *J. Cell. Physiol.* **121:**263–274
43. Sweadner, K.J., Feschenko, M.S. 2001. Predicted location and limited accessibility of protein kinase A phosphorylation site on Na,K-ATPase. *Am. J. Physiol.* **280:**C1017–C1026
44. Toyoshima, C., Nakasako, M., Nomura, H., Ogawa, H. 2000. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature* **405:**647–655
45. Uyttersprot, N., Costagliola, S., Miot, F. 1998. A new tool for efficient transfection of dog and human thyrocytes in primary culture. *Mol. Cell. Endocrinol.* **142:**35–39
46. Vara, J.A., Portela, A., Ortin, J., Jimenez, A. 1986. Expression in mammalian cells of a gene from *Streptomyces alboniger* conferring puromycin resistance. *Nucleic Acids Res.* **14:**4617–4624