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RESEARCH PAPER

Localization of intracellular compartments that exchange Na,K-ATPase molecules with the plasma membrane in a hormone-dependent manner

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Background and Purpose: Dopamine is a major regulator of sodium reabsorption in proximal tubule epithelia. By binding to D1-receptors, dopamine induces endocytosis of plasma membrane Na,K-ATPase, resulting in a reduced capacity of the cells to transport sodium, thus contributing to natriuresis. We have previously demonstrated several aspects of the molecular mechanism by which dopamine induces Na,K-ATPase endocytosis; however, the location of intracellular compartments containing Na,K-ATPase molecules has not been identified.
Experimental approach: In this study, we used different approaches to determine the localization of Na,K-ATPase-containing intracellular compartments. By expression of fluorescent-tagged Na,K-ATPase molecules in opossum kidney cells, a cell culture model of proximal tubule epithelia, we used fluorescence microscopy to determine cellular distribution of the fluorescent molecules and the effects of dopamine on this distribution. By labelling cell surface Na,K-ATPase molecules from the cell exterior with either biotin or an epitope-tagged antibody, we determined the localization of the tagged Na,K-ATPase molecules after endocytosis induced by dopamine.
Key results: In cells expressing fluorescent-tagged Na,K-ATPase molecules, there were intracellular compartments containing Na,K-ATPase molecules. These compartments were in very close proximity to the plasma membrane. Upon treatment of the cells with dopamine, the fluorescence labelling of these compartments was increased. The labelling of these compartments was also observed when the endocytosis of biotin- or antibody-tagged plasma membrane Na,K-ATPase molecules was induced by dopamine.
Conclusions and Implications: The intracellular compartments containing Na,K-ATPase molecules are located just underneath the plasma membrane.
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Keywords: Na,K-ATPase, dopamine, endocytosis, proximal tubule, phosphorylation, fluorescence microscopy

Abbreviations: CFP, cyan fluorescent protein; GFP, green fluorescent protein; OK, opossum kidney; PBSS, phosphate-buffered saline containing 1.2% sucrose; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; YFP, yellow fluorescent protein

Introduction

The regulation of renal sodium reabsorption is a major determinant of blood pressure (Bertorello and Katz, 1993; Bacic *et al*., 2003; Jose *et al*., 2003; McDonough and Biemesderfer, 2003). Impaired hormonal regulation of Na,K-ATPase activity in renal tubules has been linked to the development of high blood pressure (Hussain and Lokhandwala, 1998; Aperia, 2000; Efendiev *et al*., 2004). The Na,K-ATPase, located within the basolateral membrane of tubular epithelial cells, maintains a transmembrane concentration gradient for sodium, ensuring the net reabsorption of this cation (Féraille and Doucet, 2001; Kaplan, 2002). The antagonistic short-term actions of natriuretic and antinatriuretic hormones on the Na,K-ATPase activity play an important role in the control of net sodium excretion (Aperia *et al*., 1994; Féraille and Doucet, 2001). We have demonstrated in opossum kidney (OK) cells, a cell culture model of proximal tubule epithelia (Malstrom *et al*., 1987; Nash *et al*., 1993; Guimaraes *et al*., 1997), that dopamine inhibits and angiotensin II stimulates Na,K-ATPase activity (Efendiev *et al*., 1999, 2002b, 2003; Budu *et al*., 2002.). These effects are mediated by protein kinase C (PKC)-dependent phosphorylation of serine residues within the Na,K-ATPase α-subunit N-terminus (Efendiev *et al*., 1999, 2000).

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Dopamine treatment of OK cells results in phosphorylation of α1 Ser-18, and this is essential for inhibition of Na,K-ATPase activity (Chibalin *et al*., 1998a, 1999; Efendiev *et al*., 2002a). Upon phosphorylation, Na,K-ATPase molecules are internalized by endocytosis, and this results in a lower Na,K-ATPase activity at the cell membrane (Chibalin *et al*., 1999). On the other hand, angiotensin II treatment of OK cells results in phosphorylation of both α1 Ser-11 and Ser-18, and the presence of either of these amino acids is essential for angiotensin II-dependent stimulation of Na,K-ATPase (Efendiev *et al*., 1999, 2000). Increased Na,K-ATPase activity is the result of recruitment of Na,K-ATPase molecules from intracellular compartments to the plasma membrane (Efendiev *et al*., 2000; Budu *et al*., 2002). It has been described that the apical Na/H exchanger, which together with the Na,K-ATPase is responsible for proximal tubule sodium reabsorption, is also regulated in renal proximal tubules by dopamine-dependent endocytosis (Bacic *et al*., 2003; McDonough and Biemesderfer, 2003).

We have demonstrated that PKC-β and ζ are the isoforms responsible for the opposing effects of angiotensin II and dopamine, respectively, on proximal tubule Na,K-ATPase activity (Efendiev *et al*., 1999), and that Ser-18 is the only amino-acid residue within the rat α1-subunit that is phosphorylated and is relevant for dopamine-dependent regulation of proximal tubule Na,K-ATPase activity (Efendiev *et al*., 2000; Efendiev *et al*., 2002a). Hormone-dependent phosphorylation of serine residues located in the N-terminus of α1 triggers the mechanism by which phosphorylated Na,K-ATPase molecules are either retrieved from or recruited to the plasma membrane. This mechanism assumes that the epithelial cells must contain intracellular compartments that exchange Na,K-ATPase molecules with the pool of molecules present in the plasma membrane. By cell fractionation, it has been observed that about 30% of the cellular Na,K-ATPase may be contained in intracellular compartments that have the potential capacity to exchange molecules with the plasma membrane pool (Chibalin *et al*., 1999). However, attempts to localize the intracellular compartments containing Na,K-ATPase molecules have so far failed.

In several of our previous publications, we have demonstrated that dopamine induces the endocytosis of Na,K-ATPase molecules (reviewed in Pedemonte and Bertorello, 2001; Pedemonte *et al*., 2005). We have quantified previously the magnitude of the endocytosis, and described several aspects of the molecular mechanism that is involved in the dopamine-dependent translocation of Na,K-ATPase molecules. Thus, the present studies were not to measure or to demonstrate the endocytosis of Na,K-ATPase molecules, which has been already demonstrated, but to determine the cellular localization of the intracellular compartments containing Na,K-ATPase molecules.

Methods

Cell culture and transfection

Although the Na,K-ATPase may be associated with other regulatory proteins, the minimal functional unit is composed of the α- and β-subunits (Kaplan, 2002). Several different isoforms of these subunits are expressed in different mammalian tissues. As the kidney mainly expresses the α1 and β1 polypeptides (Lingrel *et al*., 2003), in our transfection experiments, we used cells expressing only the α1 and/or β1 isoforms. OK cells stably expressing the wild-type rat Na,K-ATPase α1 (α1-wt), or a mutant of this protein (α1-S18A), were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics. Plasmid preparation, site-directed mutagenesis and stable expression of Na,K-ATPase α1 were performed as described previously (Pedemonte *et al*., 1997a, b). Cells expressing Na,K-ATPase α1 were selected with 3 μM ouabain (Pedemonte *et al*., 1997a, b). For fluorescence determinations, cells were transfected with the cDNAs of α1 fused to yellow fluorescent protein (YFP) at the N-terminal end (YFP-α1) and/or β1 fused to cyan fluorescent protein (CFP) at the C-terminal end (β1-CFP) (Efendiev *et al*., 2006). For expression of β1-CFP, wild-type OK cells were transfected with expression vectors for both rat α1 and β1-CFP (the latter containing the neo-gene conferring resistance to G418), and selected both with 3 μM ouabain and 0.8 mg ml^{-1} G418 (Efendiev *et al*., 2006). Before treatment with hormones, cells were incubated for 30 min in the same culture medium without serum and buffered at pH 7.5 with 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid).

Some of the experiments were performed with cells expressing the S18A mutant of α1. This mutation does not affect the basic Na,K-ATPase activity, but dopamine-dependent phosphorylation of Na,K-ATPase molecules containing this mutant does not occur because the site of phosphorylation (Ser-18) has been eliminated (Pedemonte *et al*., 1997a, b). Therefore, dopamine cannot induce the endocytosis and inhibition of Na,K-ATPase molecules containing the α1-S18A mutant.

Cell treatment

The optimal concentrations of the reagents and the extension of the treatments used in these experiments were as determined previously (Efendiev *et al*., 2000, 2002b; Efendiev and Pedemonte, 2006). Cells attached to glass coverslips were incubated with 5 μM monensin for 30 min and then with 1 μM dopamine for 10 min at room temperature. The cells were washed twice with phosphate-buffered saline containing 1.2% sucrose (PBSS), fixed with freshly prepared 4% paraformaldehyde in PBSS for 10 min at 4°C, washed twice with PBSS, permeabilized with 0.2% Triton X-100 in PBSS for 20 min and washed again twice with PBSS. Then, the coverslips were mounted on glass slides using mowiol (EMD BioSciences, San Diego, CA, USA). In some of the experiments, before and during the treatment with hormones, the cells were incubated with 2 μM chelerythrine chloride for 30 min to inhibit PKC activity or with 100 nM wortmannin for 20 min to inhibit phosphoinositide 3-kinase (PI3 K) activity.

For biotinylation experiments, the cells on coverslips were incubated with 10 mM Tris–HCl (pH 7.5), 2 mM $CaCl_2$, 150 mM NaCl and 1.5 mg ml^{-1} Sulfo-NHS-Biotin for 1 h at 4°C. After washing the cells with ice-cold PBSS to eliminate the excess Sulfo-NHS-Biotin, they were treated with 1 μM

dopamine for 10 min at room temperature. Then, the cells were fixed as indicated above. This opens the plasma membrane and exposes intracellular compartments. To detect the presence of biotin, the cells were treated with 10 $\mu g\,ml^{-1}$ Avidin-Texas Red for 1 h. Finally, the cells were washed with ice-cold PBSS and the coverslips were mounted on glass slides.

For the experiments using an antibody to label cell surface Na,K-ATPase molecules, OK cells co-expressing α1-wt and β1-CFP were treated for 1 h with 5 $\mu g\,ml^{-1}$ anti-green fluorescent protein (GFP)-Alexa Fluor 594 antibody. After washing off the excess antibody, the cells were treated with 1 μM dopamine for 10 min at room temperature. Then, the cells were fixed as indicated above. Finally, the cells were washed with ice-cold PBSS and the coverslips were mounted on glass slides. Each experiment was repeated at least three times.

Optical setup of fluorescence microscopy

To obtain high-resolution three-dimensional images of the cells, the fluorescence imaging workstation consisted of an Olympus IX-81 inverted fluorescence microscope (Olympus Corp., Tokyo, Japan), equipped with $\times 60/\times 100$ oil immersion objective lens, cooled Hamamatsu ORCA-ER CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan), a halogen 100-W light source, a motorized filter and shutter, scan wizard for collection of *x*/*y* and *z* image sequences and wavelength positions over time, all controlled by the SimplePCI software (Compix, Cranberry Township, PA, USA). For all intensity measurements, the Dynamic Intensity Analysis module of SimplePCI was used. Cell fluorescence was determined using different channels with the corresponding filters.

Statistical procedures

Each experiment was repeated at least three times, and each time at least eight randomly chosen cells were analyzed. Figures show representative images for each experiment.

Materials

Ouabain, dopamine, G418, chelerythrine chloride and wortmannin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Alexa Fluor 594-tagged antibody against GFP and Avidin-Texas Red were purchased from Molecular Probes (Eugene, OR, USA). Sulfo-NHS-Biotin was obtained from Pierce (Rockford, IL, USA). Anti-Na,K-ATPase-α1 antibody was a generous gift of Drs Robert W Mercer (Washington University, St Louis, MO, USA) and Gustavo Blanco (University of Kansas Medical Center, Kansas City, KS, USA). Other reagents were of the highest quality available.

Results

The experiments were performed with OK cells, a cell culture model of proximal tubule epithelia (Malstrom *et al*., 1987; Nash *et al*., 1993; Guimaraes *et al*., 1997), expressing the rodent Na,K-ATPase α1. We have demonstrated previously that, under basal conditions, dopamine has no effect on the Na,K-ATPase activity and that dopamine-dependent inhibition of this activity requires a slight increase in intracellular sodium concentration. Thus, in all of the experiments, cells were treated for 30 min with 5 μM monensin (to increase intracellular sodium concentration from 9 to 19 mM) and then with 1 μM dopamine for 10 min (Efendiev *et al*., 2002b). The small increase in intracellular sodium produced by monensin does not affect the content of the plasma membrane pool of Na,K-ATPase molecules (Efendiev and Pedemonte, 2005).

Fluorescence determinations

In the first set of experiments, we used OK cells expressing the rat Na,K-ATPase α1- and β1-subunits with YFP fused to the α1 N-terminus (YFP-α1) and CFP fused to the β1 C-terminus (β1-CFP). Thus, Na,K-ATPase molecules would have fused YFP molecules in the intracellular face of the plasma membrane and fused CFP molecules in the external face of the plasma membrane. Cells expressing YFP-α1 were selected with 3 μM ouabain. We have demonstrated previously that, under this selection condition, endogenous Na,K-ATPase molecules are inhibited, because they are sensitive to this level of ouabain (Pedemonte *et al*., 1997a). As the exogenously introduced cDNAs express rat Na,K-ATPase molecules that are resistant to 3 μM ouabain, the expressed Na,K-ATPase molecules substitutes the endogenous ouabain-inhibited activity. The cell regulates the expression of the rodent Na,K-ATPase α1 and β1 to produce just enough active Na,K-ATPase molecules to compensate for the endogenous inhibited activity. We, and other researchers, have observed that the level of cellular Na,K-ATPase activity in transfected cells is the same as the one observed in non-transfected cells (Pedemonte *et al*., 1997a, b; Duran *et al*., 2004).

The following determinations were performed by acquiring fluorescence images of the cells using a filter that would excite the YFP fluorophore (Figure 1). To ensure that the fluorescence we attribute to intracellular compartments is really produced by fluorophores in cytosolic compartments, rather than by those in the plasma membrane of the top or bottom sections of the cell, we performed determinations to acquire *x*/*y* section images of the cell at different depth (*z*-stack). The microscope objective was initially focused to determine the top and bottom sections, in which fluorescence was barely detectable. The depth (*z* axis distance between top and bottom sections) of OK cells estimated this way was 9 ± 1 μm. The microscope was then set up to capture seven sequential *x*/*y* section images from top to bottom. Section #4, being the closest to the middle of the cell, was arbitrarily chosen as the zero point of reference. Figure 1 shows fluorescent images taken at three different sections of the cells: section #2, depth $= +3$ μm, section #4, depth $= 0$ and section #6, depth $= -3$ μm.

In cells not treated with dopamine, the fluorescence was concentrated at the plasma membrane (Figures 1b–d). However, the thickness of fluorescence was not uniform around the cell, there were regions in which the fluorescence was thicker and spots of fluorescence appeared to be in very close proximity but separated from the plasma membrane (indicated by arrows in Figures 1b–d). In cells that had been

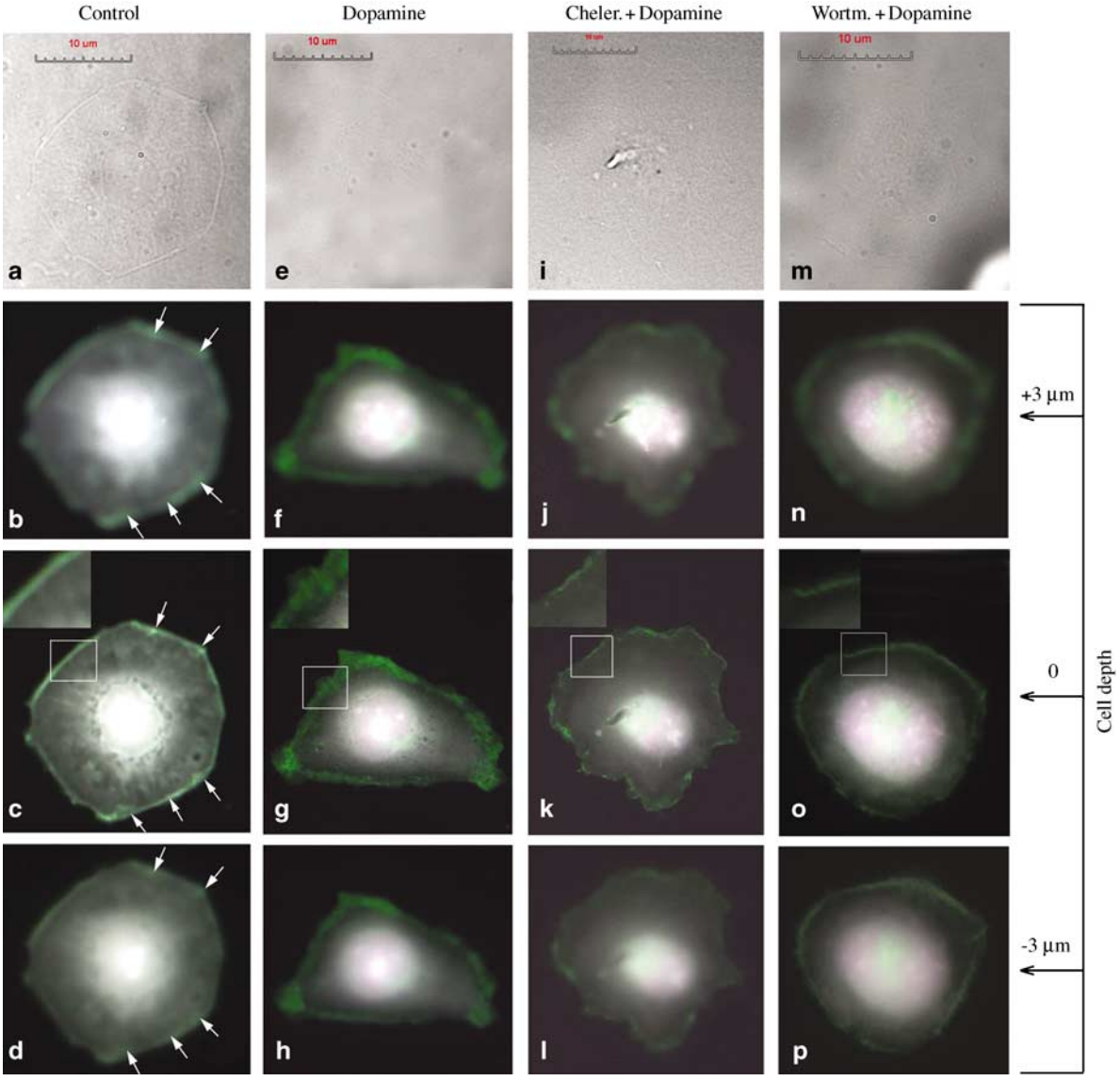

Figure 1 Localization of Na,K-ATPase molecules tagged with fluorescent markers. OK cells were stably co-transfected with Na,K-ATPase α1 tagged with YFP at its N-terminus (YFP-α1) and β1 tagged with CFP at its C-terminus (β1-CFP). Cells were treated (or not) with chelerythrine chloride, wortmannin and dopamine (or vehicle), as indicated in Materials and methods section. Brightfield images: (**a**, **e**, **i** and **m**) fluorescence images: Images were acquired using a 500 nm excitation/535 nm emission filter at $\times$ 60 magnification as described in the text. Images (**c**, **g**, **k** and **o**) correspond to the cell middle section. Then, images were acquired at cross-sections that are 3 μm up (images **b**, **f**, **j** and **n**), and 3 μm down from the middle cross-section (images **d**, **h**, **l** and **p**). Treatments: (**a–d**) control; (**e–h**) dopamine; (**i–l**) chelerythrine chloride and dopamine; (**m–p**) wortmannin and dopamine. Insets, higher magnification images of small regions of the cell (white rectangles). Bar, 10 μm. The arrows on images (**b–d**) indicate regions in which the fluorescence is thicker and spots of fluorescence appear to be in close proximity but separated from the plasma membrane. OK, opossum kidney.

treated with dopamine, there was no dramatic change in the pattern of cellular distribution of the fluorescence. However, regions in which the fluorescence was thicker with spots separated from the plasma membrane were more abundant in cells treated with dopamine. In cells that had been treated with the PKC inhibitor chelerythrine chloride before being treated with dopamine (Figures 1j–l), the dopamine-dependent thickening of the fluorescence was not as evident as in cells treated with dopamine alone. The dopamine-dependent thickening of the fluorescence was also reduced in cells that had been treated with the PI3K inhibitor wortmannin before being treated with dopamine (Figures 1n–p). The fluorescence thickening produced by dopamine and the inhibition of this effect by chelerythrine chloride or wortmannin treatments was observed at the three different levels of the *z* axis in which the determinations of fluorescence were performed (Figure 1). The inset pictures shown in Figure 1 were arbitrarily selected to illustrate the effects we describe above; they are not meant to show changes produced in the same cell, as each treatment was performed in separate cells.

Pictures in Figure 1 are bright in the region of the cell nucleus. The same cell preparations described in Figure 1 were also used to acquire fluorescence images using a 594/

620 nm filter, which should produce very low excitation of either YFP or CFP. Consistently, the fluorescence around the plasma membrane was greatly reduced (or eliminated), but the brightness around the nucleus was as intense as illustrated in Figure 1 (data not shown). These results clearly indicate that (1) the fluorescence of YFP and CFP was restricted to cell areas at or close to the plasma membrane and (2) the brightness around the nucleus is not produced by the fluorescence of either YFP or CFP.

In the second set of experiments, we used cell membrane protein biotinylation, as described previously in our publications (Efendiev *et al*., 2000, 2003, 2004, 2005). OK cells expressing the wild-type rat Na,K-ATPase α1 (α1-wt) were treated with NHS-biotin. As this reagent cannot cross the plasma membrane, and labelling is performed at 4°C, which impedes endocytosis, NHS-biotin would only label proteins with $-NH_2$ groups exposed at the external face of the plasma membrane. Na,K-ATPase in intracellular compartments would not be biotinylated. After biotinylation, control and dopamine-treated cells were fixed and treated with Avidin-Texas Red. This is a fluorescent reagent that specifically binds to biotin. In control cells, the fluorescence produced by Texas Red was restricted to the external contour of the cell (Figure 2a). In cells that had been treated with dopamine, the fluorescence was more diffuse and thicker than in control cells. As illustrated in Figure 2b, there were regions in which spots of fluorescence appear to be very close but separated from the plasma membrane. However, the biotin treatment should have produced the biotinylation of all of the plasma membrane proteins (not only Na,K-ATPase) containing $-NH_2$ groups exposed at the external face of the plasma membrane. Thus, the dopamine-dependent thickening of the plasma membrane observed in Figure 2b could have been due not only to endocytosis of Na,K-ATPase molecules, but also to endocytosis of other biotinylated plasma membrane proteins. To differentiate the effect of dopamine on Na,K-ATPase molecules, the determinations were repeated in cells that have been transfected with the Na,K-ATPase α1-S18A mutant, which cannot be phosphorylated in a dopamine-dependent manner (Chibalin *et al*., 1998a; Efendiev *et al*., 2003; Efendiev and Pedemonte, 2006). We have demonstrated that dopamine induces the phosphorylation of α1 Ser-18, and that this phosphorylation is essential for the dopamine-induced endocytosis of Na,K-ATPase molecules (Chibalin *et al*., 1999). Thus, Figure 2d should display the dopamine-dependent internalization of biotinylated proteins, other than Na,K-ATPase molecules; however, no significant change with respect to the control (Figure 2c) was observed. Therefore, the difference between Figures 2b and d illustrates the internalization of biotinylated Na,K-ATPase molecules, exclusively.

In the third set of experiments, we used an approach similar to that described by Tamkun and Fambrough (1986) to demonstrate the presence of intracellular compartments containing Na,K-ATPase molecules in chicken neurons. OK cells, expressing β1-CFP, were treated with an anti-GFP-Alexa Fluor 594 antibody. This antibody was raised against an epitope that is conserved for both GFP and CFP. Under the experimental conditions, the antibody only attached to CFP molecules that are exposed in the external face of the plasma membrane. As the antibody cannot cross the plasma membrane, and labeling is performed at 4°C, which impedes endocytosis, Na,K-ATPase molecules in intracellular compartments did not react with the antibody. The excess antibody was washed out, and the cells were treated with dopamine. In cells not treated with dopamine, the fluorescence of Alexa Fluor was restricted to the plasma membrane (Figure 3a). The same pattern of fluorescence was observed in cells treated with monensin (Figure 3b). However, in cells treated with dopamine, the fluorescence is more diffuse and spots of fluorescence appear to be in close proximity but separated from the plasma membrane (Figure 3c). This thickening of the fluorescence appeared to be greatly reduced when the cells were treated with either the PKC inhibitor chelerythrine chloride or the PI3K inhibitor wortmannin (Figures 3d and e).

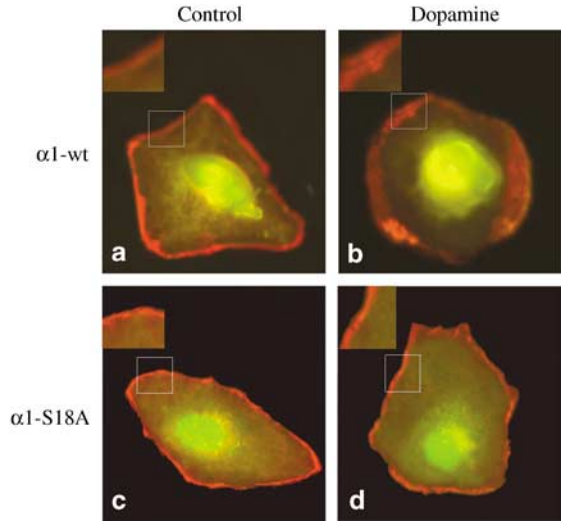

Figure 2 Cell surface protein biotinylation. Cells expressing the Na,K-ATPase wild-type α1 were labelled with Sulfo-NHS-Biotin under conditions in which only the cell membrane proteins would be labelled. After treatment with dopamine (or vehicle), the cells were fixed as indicated in Materials and methods section. The presence of biotin was detected by treatment with 10 μg/ml Avidin-Texas Red. Fluorescence images were acquired through a 594/620 nm filter at $\times$60 magnification. Treatments: (**a** and **c**) control; (**b** and **d**) dopamine. Cell lines: (**a** and **b**) α1-wt; (**c** and **d**) α1-S18A. Insets, higher magnification images of small regions of the cell (white rectangles).

Discussion

We have shown previously that activation of dopamine receptors inhibits Na,K-ATPase activity in renal epithelial cells and that this inhibition was associated with endocytosis of Na,K-ATPase molecules (Done *et al*., 2002; Efendiev *et al*., 2002a). Internalization occurs via clathrin vesicle formation and sequential transport into early and late endosomes (Chibalin *et al*., 1999; Ogimoto *et al*., 2000). On the other hand, angiotensin II induces the recruitment of Na,K-ATPase molecules to the plasma membrane resulting in an increased cellular Na,K-ATPase activity (Efendiev *et al*., 2003). These observations imply the existence of an intracellular pool of Na,K-ATPase molecules that, in a hormone-dependent

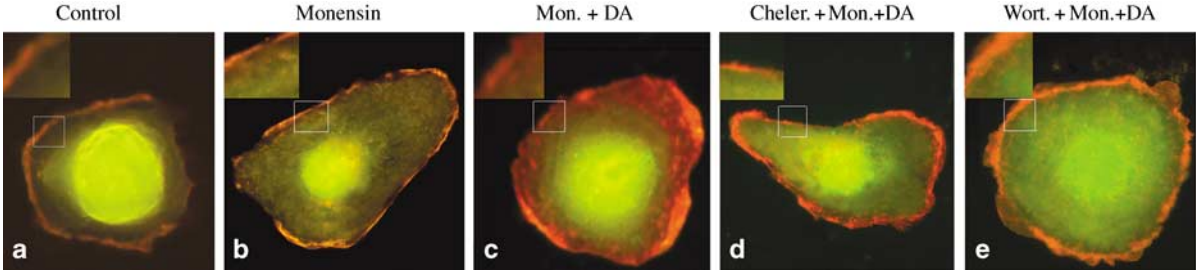

Figure 3 Cell surface Na,K-ATPase labeling with a specific antibody. OK cells co-expressing α1-wt and β1-CFP were treated anti-GFP-Alexa Fluor 594 antibody. Under the experimental conditions, the anti-GFP antibody would attach only to CFP moieties exposed in the external face of the plasma membrane. After treatment with dopamine (or vehicle), the cells were fixed as indicated in Materials and methods section. Fluorescence images were acquired through a 594/620 nm filter at $\times$60 magnification. Treatments: (**a**) control; (**b**) monensin; (**c**) monensin plus dopamine; (**d**) chelerythrine chloride plus monensin plus dopamine; (**e**) wortmannin plus monensin plus dopamine. Insets, higher magnification images of small regions of the cell (white rectangles). OK, opossum kidney.

manner, exchanges molecules with the plasma membrane Na,K-ATPase pool. Cellular fractionation experiments suggested that the intracellular pool may contain about 30% of the total cellular Na,K-ATPase content (Chibalin *et al*., 1999). However, previous attempts to identify the cellular location of the intracellular Na,K-ATPase-containing compartments failed. Reviewing images from confocal microscopy we performed as part of earlier studies, we observed that cell treatment with dopamine appeared to produce a thickening of the fluorescence associated to the plasma membrane Na,K-ATPase (an example of this is illustrated in Figure 3b of Ogimoto *et al*., 2000). Thus, the studies described in this report were undertaken to test the hypothesis that the Na,K-ATPase intracellular compartments are located just underneath the plasma membrane.

Using cells expressing fluorescent-tagged Na,K-ATPase molecules we observed that, although most of the fluorescence appears to be associated with the plasma membrane, the labelling was not uniform. There are regions in which the fluorescence is sharp, and others in which the labelling is broader, patchier and spots of fluorescence appear to be very close but separated from the plasma membrane (Figures 1b–d). In cells that had been treated with dopamine, an enhancement of the level of fluorescence just under the plasma membrane was observed, which may reflect the translocation of some of the plasma membrane Na,K-ATPase molecules to intracellular compartments (Figures 1f–h). This effect was reduced in cells that were pre-treated with the PKC inhibitor chelerythrine chloride (Figures 1j–l), which is consistent with our previous observation that the dopamine-induced endocytosis of Na,K-ATPase molecules is a direct result of α1 phosphorylation by PKC (Chibalin *et al*., 1998a). We have demonstrated previously that, on phosphorylation of α1 Ser-18, PI3K binds to a polyproline domain in the Na,K-ATPase α1 located between amino acids $TPPPTTP^{82}$ at the N-terminus of α1, and that this results in activation of PI3K (Yudowski *et al*., 2000). Inhibition of PI3K by treatment of the cells with wortmannin, prevented the dopamine-dependent endocytosis of Na,K-ATPase molecules (Chibalin *et al*., 1998b). Consistent with this, we observed that the thickening of the fluorescence induced by dopamine was reduced by treating the cells with wortmannin (Figures 1n–p).

In cells, whose plasma membrane proteins were biotinylated, dopamine also induced a thickening of the fluorescence associated with the plasma membrane, reflecting the translocation of biotinylated Na,K-ATPase molecules to intracellular compartments that are just underneath the plasma membrane (Figure 2b). This effect was not observed in cells expressing the S18A mutant of α1 (Figures 2c and d), which prevents the dopamine-dependent endocytosis of Na,K-ATPase molecules. A similar effect of dopamine was observed in cells whose plasma membrane Na,K-ATPase molecules were labeled with a specific antibody (Figure 3).

Using cell fractionation, we have demonstrated previously that, under basal conditions, the distribution of Na,K-ATPase was about 20% in the lysosomes, mitochondria, and endoplasmic reticulum fraction, about 50% in the plasma membrane enriched fraction and about 30% in the endosome-enriched fraction (Chibalin *et al*., 1999). Upon treatment with dopamine, the amount of Na,K-ATPase in the membrane-enriched fraction was reduced to about 30% and the endosome-enriched fraction was increased to about 50%. Therefore, although it does not seem obvious in the images illustrated in Figures 1b–d, under basal conditions, 30% of the cellular content of Na,K-ATPase molecules must be in compartments that are just underneath the plasma membrane. On the basis of fractionation experiments, dopamine does not produce a major redistribution of Na,K-ATPase molecules. This is consistent with the small changes of fluorescence localization, illustrated in Figure 1.

Dopamine-dependent endocytosis of Na,K-ATPase molecules in renal epithelial cells requires the PKC-mediated phosphorylation of Na,K-ATPase α1 and the stimulation of PI3K. Thus, the use of the PKC and PI3K inhibitors, chelerythrine chloride and wortmannin, respectively, provides a convenient method to test the specificity of the dopamine action. It can be argued that the change of pattern of Na,K-ATPase localization demonstrated in Figures 1–3 may be due to PKC-induced actin cytoskeleton rearrangement, or other morphological events at the plasma membrane, rather than Na,K-ATPase endocytosis. The results from two different sets of experiment controls we performed demonstrate that this is not the case. First, the fluorescent pattern change is not observed in cells expressing the Na,K-ATPase containing the α1-S18A mutant, which cannot be phosphorylated by PKC. Second, the fluorescent pattern change is significantly prevented by treating the cells with wortmannin, which at the concentration used is a specific

inhibitor of PI3K (Chibalin *et al*., 1999). We have demonstrated that dopamine induces the activation of PI3K through the binding of this protein to the Na,K-ATPase, and that this is prevented by treating the cells with wortmannin (Chibalin *et al*., 1999). Treatment of the cells with wortmannin prevents the endocytosis of Na,K-ATPase molecules. The results of these control experiments also suggest that the fluorescence pattern change induced by dopamine is not due to some non-specific effects (like membrane ruffling or other unknown artifact) or to constitutive endocytosis. It should be noted that the effect of dopamine is an acute effect produced in a few minutes; thus, the effects observed are not due to delivery of newly synthesized proteins to the plasma membrane. The fact that the fluorescent thickening at the plasma membrane is not observed when cells expressing the α1-S18A mutant are treated with dopamine is strong evidence of the causal link between the specific phosphorylation of α1 and the translocation of the fluorescent Na,K-ATPase molecules to compartments that are just beneath the plasma membrane. This is consistent with our previous observations that dopamine induces the endocytosis of Na,K-ATPase molecules.

Other plasma membrane transporter proteins, like aquaporin, the Na/H exchanger and the Na/Pi exchanger, are hormonally regulated by shuttling molecules between intracellular compartments and the plasma membrane (Bacic *et al*., 2003, 2005; McDonough and Biemesderfer, 2003; Bobulescu *et al*., 2005; Nejsum *et al*., 2005; Fuster *et al*., 2007).

In conclusion, we have presented evidence, in several of our previous publications, of the existence of intracellular compartments that, under hormonal control, exchange Na,K-ATPase with the plasma membrane (reviewed in Pedemonte *et al*., 2005). However, the localization of Na, K-ATPase-containing compartments has been difficult to identify. Now, using different techniques and approaches, we show that the difficulty on identifying these compartments was due to the fact that they are located just underneath the plasma membrane.

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Conflict of interest

The authors state no conflict of interest.

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