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Hormonal-dependent recruitment of Na^+,K^+-ATPase to the plasmalemma is mediated by PKC_β and modulated by $[Na^+]_i$

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1 The present study demonstrates that stimulation of hormonal receptors of proximal tubule cells with the serotonin-agonist 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT) induces an augmentation of Na^+,K^+-ATPase activity that results from the recruitment of enzyme molecules to the plasmalemma.

2 Cells expressing the rodent wild-type Na^+,K^+-ATPase α-subunit had the same basal Na^+,K^+-ATPase activity as cells expressing the α-subunit S11A or S18A mutants, but stimulation of Na^+,K^+-ATPase activity was completely abolished in either mutant.

3 8-OH-DPAT treatment of OK cells led to PKC_β-dependent phosphorylation of the α-subunit Ser-11 and Ser-18 residues, and determination of enzyme activity with the S11A and S18A mutants indicated that both residues are essential for the agonist-dependent stimulation of Na^+,K^+-ATPase activity.

4 When cells were treated with both dopamine and 8-OH-DPAT, an activation of Na^+,K^+-ATPase was observed at basal intracellular sodium concentration (~9 mM), and this activation was gradually reduced and became a significant inhibition as the concentration of intracellular sodium gradually increased from 9 to 19 mM. Thus, besides the antagonistic effects of dopamine and 8-OH-DPAT, intracellular sodium modulates whether an activation or an inhibition of Na^+,K^+-ATPase is produced.

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Abbreviations: 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino) tetraline; DMEM, Dulbecco's modified Eagle's medium; $[Na^+]_i$, intracellular sodium concentration; OK, opossum kidney; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SBFI, sodium-binding benzofuran-isophthalate

Introduction

The Na^+,K^+-ATPase, located within the basolateral membrane of tubule epithelial cells, maintains a transmembrane concentration gradient for sodium, ensuring the net reabsorption of this cation. Short-term regulation of kidney Na^+,K^+-ATPase by hormones and intracellular second messengers contributes to the ability of the kidney to adjust sodium reabsorption (Bertorello & Katz, 1993; Hussain & Lokhandwala, 1998; Aperia, 2000; Therien & Blostein, 2000; Féraille & Doucet, 2001; Pedemonte & Bertorello, 2001). Intrarenal dopamine has a diverse action but one principal effect is the inhibition of proximal tubule Na^+,K^+-ATPase (Bertorello & Katz, 1993; Hussain & Lokhandwala, 1998; Aperia, 2000). Decreased Na^+,K^+-ATPase activity induced by dopamine is partly responsible for reduced sodium reabsorption during a high salt diet, and impaired regulation of the Na^+,K^+-ATPase activity in renal tubules has been linked to the development of high blood pressure (Bertorello & Katz, 1993; Hussain & Lokhandwala, 1998; Aperia, 2000).

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It has been suggested that the interplay between the antagonistic actions of the natriuretic dopamine and the antinatriuretic serotonin plays a major role in renal sodium and water excretion (Soares da Silva *et al*., 1996b; Vieira-Coelho *et al*., 1997). Both amines modulate urinary sodium excretion without affecting renal haemodynamics (Itskowitz *et al*., 1988; Li Kam Wa *et al*., 1996; Siragy *et al*., 1989). In the kidney, dopamine and serotonin originate in the same renal proximal convoluted tubule epithelial cells. The synthesis and degradation of the two intrarenal hormones have several common steps and it has been suggested that the synthesis of the two amines by the proximal epithelial cells may be mutually regulated (Soares da Silva *et al*., 1996a). The precursors of intrarenal dopamine and serotonin, L-DOPA and L-5-HTP respectively, enter the epithelial cells of proximal convoluted tubules through a common transporter (Soares da Silva *et al*., 1995). Furthermore, the two amines share a common synthetic pathway: decarboxylation of their respective amino acid precursors, and a common metabolic pathway: oxidation by type A monoamine oxidase (Soares da Silva & Pinto-do-O, 1996). In contrast to dopamine, serotonin of renal origin is antinatriuretic. Since the energy

for tubular sodium reabsorption is supplied by the Na^+,K^+-ATPase, it might then be suggested that stimulation of tubular sodium absorption by serotonin would involve activation of the Na^+,K^+-ATPase. Indeed, Soares da Silva *et al.* (1996b) demonstrated that stimulation of serotonin receptors with the specific agonist 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT) induced an augmentation of Na^+,K^+-ATPase activity. However, the molecular mechanism involved in the activation of Na^+,K^+-ATPase by stimulation of serotonin receptors remains unknown and is the object of the present study.

Methods

Cell culture and transfection

Opossum kidney (OK) cells were maintained at 37°C (10% CO_2) in Dulbecco's modified Eagle medium with 10% calf serum and antibiotics (DMEM-10). Mutants of rodent Na^+,K^+-ATPase α1 subunit cDNA were prepared, as previously described (Pedemonte *et al.*, 1997a, b; Chibalin *et al.*, 1999; Efendiev *et al.*, 2000), from a plasmid containing the wild-type α1-subunit sequence and complementary oligonucleotides containing the desired change. Briefly, annealed plasmid and oligonucleotides were subjected to PCR amplification with *Pfu* polymerase, followed by restriction of the original wild-type template with *Dpn I*. After transformation of bacteria, the recovered mutant plasmids were evaluated by restriction analysis and direct sequencing of the altered region. Plasmids containing the wild-type and mutated α1 subunit cDNAs were transfected into OK cells using LipofectAmine liposomes (Gibco/Invitrogen, Carlsbad, CA, U.S.A.), as previously described (Pedemonte *et al.*, 1997a, b; Chibalin *et al.*, 1999; Efendiev *et al.*, 2000). Selection for cells expressing the highest level of rodent α1 subunit was achieved by exposing the cells to a medium containing 3 μM ouabain. Since the endogenous Na^+-pump of OK cells is completely inhibited by this concentration of ouabain (Pedemonte *et al.*, 1997a, b; Chibalin *et al.*, 1999; Efendiev *et al.*, 2000), only successful recipients of transfected rodent α-subunit would be able to survive. Resistant colonies were expanded and maintained in DMEM-10 containing 3 μM ouabain. Experiments were performed with a mix of at least 20 independent clones for each cell line. The Na^+,K^+-ATPase of mock-transfected cells (vector alone, vector plus liposomes, or liposomes alone) had the same activity and sensitivity to ouabain as non-transfected host cells.

Determination of Rb^+-transport

Measurements of Na^+,K^+-ATPase-mediated transport by Rb^+-uptake were performed with attached cells as previously described (Pedemonte *et al.*, 1997a, b; Efendiev *et al.*, 2000). Briefly, to measure Rb^+-transport cells were transferred to serum-free DMEM containing 50 mM HEPES, pH 7.4 (DMEM-HEPES), and either 3 μM or 5 mM ouabain (incubation medium). All treatments and determinations were performed at 25°C. After treatment, a trace amount of [$^{86}Rb^+$]-RbCl was added to the cell medium. After 20 min, cells were washed four times with ice-cold saline, dissolved with SDS, and accumulated radioactivity was determined. Na^+,K^+-ATPase-mediated Rb^+-transport was calculated from the difference in tracer uptake between samples incubated in 3 μM and 5 mM ouabain. The ouabain-insensitive Rb^+-transport was 25–30% of the total Rb^+-transport measured.

Monitoring ionic changes in OK cells

Optical determinations of intracellular sodium with the sodium-binding benzofuran-isophthalate (SBFI) were performed as previously described (Pedemonte *et al.*, 1997a, b; Efendiev *et al.*, 2002). Based on the changes in intracellular sodium produced by different concentrations of monensin a linear relation between the concentration of monensin in the cell medium and the concentration of intracellular sodium was calculated (Efendiev *et al.*, 2002). The linear equation from this plot ($[Na^+]_i = (2.25 \pm 0.11)$[monensin] $10^3 + (8.92 \pm 0.71)$ mM) was used to calculate the $[Na^+]_i$ that corresponds to the concentration of monensin in the cell medium.

Other determinations and data analysis

Determination of protein concentration, labelling with NHS-biotin, phosphorylation and immunoprecipitation of the Na^+,K^+-ATPase were performed as previously described (Efendiev *et al.*, 2000). Comparisons between groups were performed by Student's *t*-test for unpaired data and one-way analysis of variance ANOVA. A P value less than 0.05 for *t*-test and less than 0.01 for ANOVA were considered significant.

Results

8-OH-DPAT stimulation of Na^+,K^+-ATPase activity is mediated by PKCβ

All experiments were performed with OK cells expressing the rodent α1 subunit (Pedemonte *et al.*, 1997a, b). OK cells are an established epithelial cell line often studied as a physiological model system of renal proximal tubule function (Malstrom *et al.*, 1987; Nash *et al.*, 1993). Treatment of the cells with the serotonin receptor agonist 8-OH-DPAT induced a concentration dependent increase in Na^+,K^+-ATPase activity (Figure 1). The stimulatory effect of 8-OH-DPAT was prevented by addition of either 10 nM or 3 μM LY333531 in the cell medium. It has been previously demonstrated that 10 nM LY333531 specifically inhibits protein kinase C β isoform (PKC_β) *in vivo* and *in vitro* (Ishii *et al.*, 1996; Kowluru *et al.*, 1998); and we have shown that LY333531 also prevented the activation of Na^+,K^+-ATPase by phorbol 12-myristate 13-acetate (PMA) (Efendiev *et al.*, 1999). At 3 μM, LY333531 inhibits all of the conventional PKC isoforms, PKC_η and PKC_ζ (Ishii *et al.*, 1996). Although the Na^+,K^+-ATPase activities at 10 nM and 3 μM LY333531 are slightly different, there is no significant difference between them (Figure 1). Therefore, these results suggest that the stimulation of Na^+,K^+-ATPase activity by 8-OH-DPAT is mediated by PKC_β.

8-OH-DPAT (3 μM) and PMA (1 μM) produced approximately the same level of Na^+,K^+-ATPase activation (Figure

2). However, the determination of Rb^+-transport in cells treated simultaneously with both PMA and 8-OH-DPAT showed that the stimulatory effects of PMA and 8-OH-DPAT on the Na^+,K^+-ATPase activity were not additive (PMA, $35\pm2\%$; 8-OH-DPAT, $35\pm3\%$; PMA plus 8-OH-DPAT, $33\pm2\%$) (Figure 2). This result suggests that PMA and 8-OH-DPAT share a common signalling pathway to activate the Na^+,K^+-ATPase. The PKC inhibitor staurosporine (0.1 μM) had no effect on the basal Na^+,K^+-ATPase activity, but prevented the stimulation of the Na^+,K^+-ATPase activity by either PMA or 8-OH-DPAT. At the concentration of 0.1 μM, staurosporine inhibits all of the PKC isoforms except PKC_ζ (Ishii *et al*., 1996). Therefore, PKC_ζ is not involved in the stimulation of Na^+,K^+-ATPase activity by 8-OH-DPAT. We have previously demonstrated that PKC_ζ is involved in the inhibition of Na^+,K^+-ATPase by dopamine, but not in the stimulation of this activity by PMA (Efendiev *et al*., 1999).

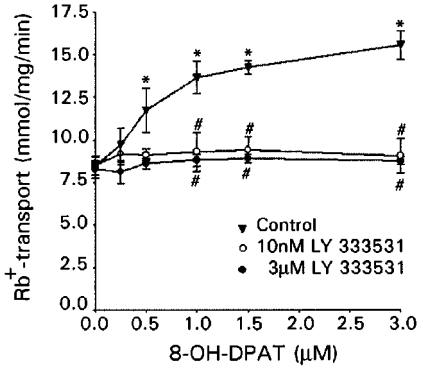

Figure 1 Effect of the PKC_β-inhibitor LY333531 on the 8-OH-DPAT-induced activation of Rb^+-transport mediated by the Na^+,K^+-ATPase. Cells were treated with either 10 nM or 3 μM LY333531 for 30 min before treatment with various concentrations of 8-OH-DPAT for 10 min. Control Rb^+-transport was measured in the absence of LY333531. The basal Na^+,K^+-ATPase activity was 8.3 ± 0.7 nmol mg min^{-1}. *Indicates significant differences with respect to the basal values. #Indicates significant differences compared to the respective control. Data were analysed by one-way analysis of variance ($P<0.01$).

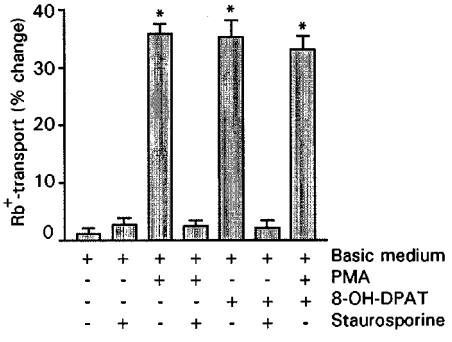

Figure 2 The stimulatory effects of PMA and 8-OH-DPAT on the Na^+,K^+-ATPase activity are not additive. Cells were treated with 1 μM PMA and/or 3 μM 8-OH-DPAT for 10 min before assay of Rb^+-transport. When indicated, cells were treated with 0.1 μM staurosporine for 30 min. The percentage of change for each experimental condition was calculated with respect to a control in the absence of 8-OH-DPAT and/or PMA. *$P<0.05$ with respect to control.

Ser-11 and Ser-18 are essential for the 8-OH-DPAT stimulation of Na^+,K^+-ATPase activity

We have previously demonstrated that dopamine- and PMA-dependent regulation of the Na^+,K^+-ATPase requires the integrity of the Na^+,K^+-ATPase α-subunit NH_2-terminus (Efendiev *et al*., 2000). Consistent with this, the activation of Na^+,K^+-ATPase by 8-OH-DPAT and PMA was not observed in molecules containing a truncated α-subunit in which the first 26 amino acids have been eliminated (Figure 3). This mutation did not affect the basal Na^+,K^+-ATPase activity. The Na^+,K^+-ATPase α-subunit amino terminus contains Ser-11 and Ser-18, which we have demonstrated are phosphorylated by PKC during the regulation of the Na^+,K^+-ATPase activity by PMA and dopamine (Efendiev *et al*., 2000). To characterize the amino acids that are involved in the activation of Na^+,K^+-ATPase by 8-OH-DPAT, we performed experiments in cells transfected with α-subunit in which the Ser-11 and Ser-18 residues have been mutated to Ala residues. The basal level of ouabain-sensitive Rb^+-transport was the same in cells transfected with wild-type and mutant rodent α-subunit cDNAs. In cells expressing the wild-type rodent α-subunit, treatment with either 8-OH-DPAT or PMA resulted in an increased level of ouabain-sensitive Rb^+-transport (Figure 3). However, substitution of either Ser-11 or Ser-18 with an alanine residue (S11A and S18A) blunted the stimulation of Rb^+-transport elicited by either PMA or 8-OH-DPAT (Figure 3). Since none of the mutations altered the basal Na^+,K^+-ATPase activity measured in the absence of either PMA or 8-OH-DPAT, these mutations have affected specifically the mechanism of Na^+,K^+-ATPase activation and not the intrinsic mechanism of Na^+,K^+-ATPase activity.

8-OH-DPAT dependent phosphorylation of the Na^+,K^+-ATPase α-subunit

The fact that Ser-11 and Ser-18 are essential amino acids for 8-OH-DPAT-dependent activation of Na^+,K^+-ATPase, and

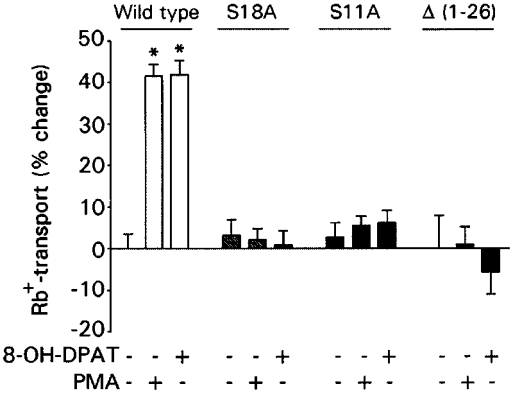

Figure 3 Both Ser-11 and Ser-18 are essential for the stimulation of Na^+,K^+-ATPase activity induced PMA or 8-OH-DPAT. The Rb^+-transport mediated by the Na^+,K^+-ATPase of cells expressing the wild-type rodent α1-subunit and three α1 mutants was determined. $\Delta(1-26)$ is the mutant in which amino acids 1–26 of the mature α1-subunit were deleted. S11A and S18A represent the activity in cells expressing the α1 mutants in which Ser-11 and Ser-18 have been substituted by alanine residues. The percentage of change for each condition was calculated with respect to a control in the absence of either 8-OH-DPAT or PMA. *$P<0.05$ with respect to control.

that this process is mediated by PKC_{β}, suggest that these residues might be phosphorylated by the 8-OH-DPAT treatment. The level of 8-OH-DPAT-dependent Na^+,K^+-ATPase α-subunit phosphorylation was measured in cells expressing the rodent wild-type and the S11A and S18A mutants of the Na^+,K^+-ATPase α-subunit. There was no difference in the basal level (without 8-OH-DPAT) of phosphorylation between the mutants and the wild-type α-subunit. Although treatment with 8-OH-DPAT produced an increased level of phosphorylation of the α-subunit in cells expressing the α-subunit wild-type and mutants, the increase of phosphorylation in S11A and S18A cells is lower than in wild-type cells (Figure 4). The reduced phosphorylation of α1 in S11A and S18A cells is consistent with the fact that one of the serine residues has been mutated to an amino acid that cannot be phosphorylated. Then, the blunted activation of Na^+,K^+-ATPase by 8-OH-DPAT in S11A or S18A cells (Figure 3) is due to the mutation of one of the serine residues. These results suggest that the activation of Na^+,K^+-ATPase by 8-OH-DPAT requires the phosphorylation of both Ser-11 and Ser-18 in the Na^+,K^+-ATPase α-subunit. Similar results were previously determined for the activation of Na^+,K^+-ATPase by PMA (Efendiev *et al*., 2000).

8-OH-DPAT stimulation of Na^+,K^+-ATPase activity is due to recruitment of Na^+,K^+-ATPase molecules to the plasma membrane

We have previously demonstrated that regulation of proximal tubule Na^+,K^+-ATPase activity by either PMA or dopamine is not due to an improved turnover of the enzyme present at the plasma membrane, but rather mediated by shuttling Na^+,K^+-ATPase molecules between the plasma membrane and intracellular storage compartments (Efendiev *et al*., 2000). As we could not use labelling with radioactive ouabain because the rodent α-subunit has a low affinity for ouabain (Asano *et al*., 1999), biotinylation of the proteins that are at the cell surface followed by immunoprecipitation of the biotinylated Na^+,K^+-ATPase molecules was used to determine the number of Na^+,K^+-ATPase molecules present in the plasma membrane. After treatment with 8-OH-DPAT, the temperature of the cell medium was reduced to 4°C to label plasma membrane proteins with NHS-biotin. The low temperature impeded the shuttling of Na^+,K^+-ATPase molecules between the plasma membrane and intracellular compartments locking the recruited Na^+,K^+-ATPase molecules at the plasma membrane during the treatment with NHS-biotin. This reagent reacts with primary amino groups and does not permeate across biological membranes; thus, protein side chains containing primary amines that are exposed to the extracellular medium were biotinylated. Then, cells were lysed and biotinylated proteins were immunoprecipitated with streptavidin bound to magnetic beads. The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto PVDF membrane, and the α-subunit of the Na^+,K^+-ATPase was identified by Western blotting with an anti-α-subunit monoclonal antibody (α1). The antibody α1 was a kind gift from Dr Robert Mercer (Washington University, St. Louis, MO, U.S.A.). The results illustrated in Figure 5 indicate a significant increase in the level of biotinylated Na^+,K^+-ATPase produced by treatment of the cells with either 8-OH-DPAT or PMA. These data suggest a ~40% increase in Na^+,K^+-ATPase molecules at the plasma membrane (Figure 5), consistent with the magnitude of the increase of Rb^+-transport illustrated in Figures 2 and 3.

Intracellular sodium modulates the stimulatory effect of 8-OH-DPAT on the Na^+,K^+-ATPase activity

We have previously demonstrated that the effects of dopamine and PMA on the Na^+,K^+-ATPase activity are dependent on the $[Na^+]_i$ (Efendiev *et al*., 2002). The sodium ionophore monensin was used to induce stable incremental

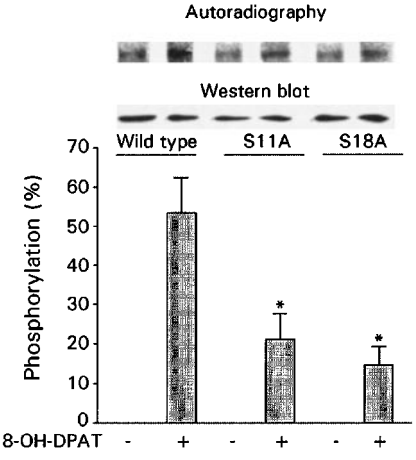

Figure 4 Phosphorylation of the α1-subunit Ser-11 and Ser-18 residues induced by 8-OH-DPAT. The effect of 8-OH-DPAT treatment on the phosphorylation of the rodent α1 subunit wild-type and the S11A and S18A mutants was determined. A representative autoradiogram and the corresponding Western blot are shown in the upper panel. The ratio of radioactivity to protein was calculated for each α1 subunit band. No significant change in the basal level of phosphorylation between mutants and wild-type α-subunit was observed. Data are presented as a percentage of 8-OH-DPAT-dependent phosphorylation with respect to the corresponding non-treated controls. $^*P<0.05$ with respect to the maximal level of 8-OH-DPAT-dependent phosphorylation of wild-type α1.

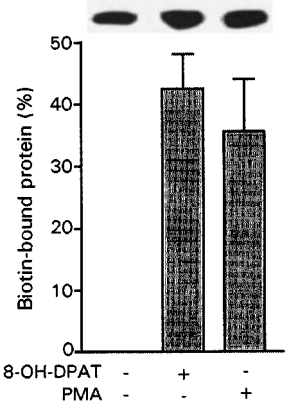

Figure 5 Binding of biotin to the plasma membrane Na^+,K^+-ATPase of OK cells transfected with the rodent α1-subunit. Plasmalemma proteins of OK cells transfected with the rodent α1 subunit were labelled by treatment with NHS-biotin. A representative experiment of the quantitation of biotin is illustrated in the upper panel. Data are presented as the percentage of biotinylation induced by either PMA or 8-OH-DPAT with respect to a non-treated control.

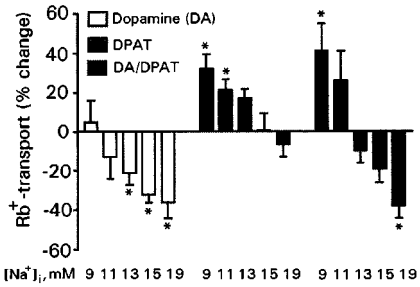

Figure 6 The intracellular Na^+ concentration modulates the effects induced by 8-OH-DPAT and dopamine on the Na^+,K^+-ATPase activity. Cells were incubated with the indicated concentrations of monensin for 30 min before treatment with 3 μM 8-OH-DPAT for 10 min and/or 1 μM dopamine for 5 min. The percentage of change for each concentration of monensin was calculated with respect to a control in the absence of 8-OH-DPAT and dopamine. $^*P<0.05$ with respect to a control.

concentrations of intracellular sodium, and these changes were monitored by digital fluorescence microscopy of cells loaded with the sodium indicator SBFI (Harootunian *et al*., 1989). Using standard concentrations of sodium, a formula that links the amount of monensin to the level of intracellular sodium was deduced (Efendiev *et al*., 2002). For the following experiments, the cells were treated with 0, 1, 2, 3 and 5 μM monensin to increase the $[Na^+]_i$ from $\sim$9 mM to $\sim$11, 13, 15 and 19 mM, respectively. Therefore, the maximal change of intracellular sodium produced was $\sim$10 mM. The changes of intracellular sodium produced by monensin were low and assumed to be within the physiological range. It is important to notice that the extracellular sodium concentration (155 mM) was not altered. We have shown that the elevated $[Na^+]_i$ elicited by monensin produced stimulation of the Na^+,K^+-ATPase activity (Efendiev *et al*., 2000). Because of this, data presented in Figure 6 represent the change in Na^+,K^+-ATPase activity produced by dopamine and/or 8-OH-DPAT in the presence of different monensin concentrations, expressed as a per cent of the Na^+,K^+-ATPase activity measured in the presence of that concentration of monensin alone. As previously reported (Efendiev *et al*., 2002), the inhibition of the Na^+,K^+-ATPase by dopamine was increased at increasing concentrations of intracellular sodium (Figure 6). On the contrary, the activation of Na^+,K^+-ATPase by 8-OH-DPAT was reduced at increasing $[Na^+]_i$. When the cells were treated with both dopamine and 8-OH-DPAT simultaneously, a stimulation of Na^+,K^+-ATPase activity was observed at basal $[Na^+]_i$ ($\sim$9 mM). However, this stimulation was reduced and became an inhibition at increasing $[Na^+]_i$. The results in Figure 6 show that there is a range of $[Na^+]_i$ (between $\sim$11 and $\sim$13 mM), in which the treatment of the cells with both agonists would not translate into any significant modification of the Na^+,K^+-ATPase activity. It is likely that at these concentrations of intracellular sodium the stimulatory effect of 8-OH-DPAT is compensated by the inhibitory effect of dopamine. The pattern of activation and inhibition produced by dopamine and 8-OH-DPAT at different $[Na^+]_i$ is the same as that we previously described for PMA (Efendiev *et al*., 2002). Thus, we have shown that PMA may either activate or inhibit the Na^+,K^+-ATPase activity depending on the $[Na^+]_i$.

Discussion

The present results demonstrate that stimulation of serotonin receptors in OK cells expressing the rodent Na^+,K^+-ATPase α1-subunit leads to activation of the Na^+,K^+-ATPase, which is due to an increased number of Na^+,K^+-ATPase molecules at the plasma membrane. Using cell surface biotin labelling, we observed a significant increment in the plasmalemmal pool of Na^+,K^+-ATPase induced by 8-OH-DPAT treatment, suggesting that the serotonin receptor agonist promoted the recruitment of Na^+,K^+-ATPase molecules to the plasma membrane. Under the conditions the experiments were performed, biotin binds exclusively molecules located in the plasma membrane, including the Na^+,K^+-ATPase. The plasma membrane pool of Na^+,K^+-ATPase is the only one contributing to ouabain-sensitive Rb^+-transport. Consistent with this, serotonin receptor activation produced a similar increase in both the plasma membrane pool of Na^+,K^+-ATPase molecules and in ouabain-sensitive Rb^+-transport.

That the stimulation of Na^+,K^+-ATPase activity by 8-OH-DPAT is due to a direct effect on the Na^+,K^+-ATPase and not a consequence of increased sodium permeability is shown by the fact that the stimulation is prevented by mutations of either Ser-11 or Ser-18, and elimination of the Na^+,K^+-ATPase α-subunit NH_2-terminus. This observation also indicates that stimulation of serotonin receptors does not activate the apical Na^+/H^+-exchanger in the specific experimental conditions used for these studies. Had either the exchanger activity or the sodium entry by any other means been increased by treatment with 8-OH-DPAT, we should have observed a stimulation of Na^+,K^+-ATPase activity in cells expressing the α-subunit mutants indicated above. Activation of serotonin receptors may not stimulate the production of cAMP, which has been linked to stimulation of the Na^+/H^+-exchanger (Clarke *et al*., 1990). We observed that LY333531 totally prevented the 8-OH-DPAT dependent activation of Na^+,K^+-ATPase. Since LY333531 does not affect protein kinase A at all, the previous result indicates that 8-OH-DPAT is not mediating any activation of Na^+,K^+-ATPase through a cAMP-dependent pathway. At the concentration used, LY333531 is a very specific inhibitor of PKC_β (Ishii *et al*., 1996; Kowluru *et al*., 1998). Therefore, the above results indicate that the 8-OH-DPAT activation of Na^+,K^+-ATPase is mediated by PKC_β.

Simultaneous phosphorylation of both Ser-11 and Ser-18 is essential for the 8-OH-DPAT dependent stimulation of Na^+,K^+-ATPase activity. Determinations of the levels of Rb^+-transport and phosphorylation with S11A and S18A mutants suggest that 8-OH-DPAT dependent stimulation of Rb^+-transport is exclusively dependent on PKC-mediated phosphorylation of Ser-11 and Ser-18. The fact that the presence of both serine residues is essential and that they are phosphorylated by stimulation of serotonin receptors suggests that phosphorylation is indeed involved in the mechanism of 8-OH-DPAT activation of Na^+,K^+-ATPase. The results presented here indicate that no phosphorylation of any residue of the α-subunit other than Ser-11 and Ser-18 is involved in the activation of Na^+,K^+-ATPase produced by stimulation of serotonin receptors. Since this activation is produced by recruitment of Na^+,K^+-ATPase molecules to the plasma membrane, phosphorylation of Ser-11 and Ser-18

are likely the triggering mechanism that leads to translocation of Na^+,K^+-ATPase molecules from intracellular compartments to the plasma membrane. In the case of PMA-induced Na^+,K^+-ATPase activation, this process occurs *via* the interaction of adaptor protein-1 with a domain of the Na^+,K^+-ATPase (Efendiev *et al*., 2000).

We have previously demonstrated that the presence of both Ser-11 and Ser-18 in the Na^+,K^+-ATPase α-subunit amino terminus is essential for Na^+,K^+-ATPase activation by PMA, and that this activation is also produced by recruitment of Na^+,K^+-ATPase molecules to the plasma membrane (Bertorello *et al*., 1999). Actually, the present results demonstrate that treatments with PMA and 8-OH-DPAT produced the same effects. The fact that the effects of PMA and 8-OH-DPAT are not additive, and that these are prevented by the PKC_β inhibitor LY333531, indicate that PMA and 8-OH-DPAT may share the same signalling pathway. The simplest explanation would be that PMA stimulates PKC_β molecules that are part of the signalling mechanism by which activation of serotonin receptors results in increased Na^+,K^+-ATPase activity.

It has been described that both serotonin and dopamine are produced by proximal tubule cells and have an antagonistic effect on sodium reabsorption (Soares da Silva *et al*., 1996b). Dopamine treatment of proximal tubule cells results in inhibition of Na^+,K^+-ATPase activity (Chibalin *et al*., 1998; 1999; Pedemonte *et al*., 1997a, b). Dopamine also acts through PKC, and only phosphorylation of Ser-18 is essential. Unlike the results with 8-OH-DPAT, the integrity of Ser-11 or its phosphorylation is not required for the effect of dopamine. Another difference between the effects of dopamine and 8-OH-DPAT is the PKC isoform involved. While the stimulatory effect of 8-OH-DPAT is blocked specifically by inhibition of PKC_β, this does not affect the dopamine-dependent inhibition of Na^+,K^+-ATPase, which is mediated by PKC_ζ (Efendiev *et al*., 1999). Inhibition of Na^+,K^+-ATPase in response to dopamine occurs by removal of Na^+,K^+-ATPase molecules from the plasma membrane (endocytosis). In contrast, activation of Na^+,K^+-ATPase in response to serotonin receptors stimulation occurs by recruitment of Na^+,K^+-ATPase molecules to the plasma membrane. We have previously demonstrated that dopamine inhibition of Na^+,K^+-ATPase is increased at higher $[Na^+]_i$ (Efendiev *et al*., 2002). The present results show that both the activation of Na^+,K^+-ATPase by stimulation of serotonin receptors and the activation/inhibition by 8-OH-DPAT/dopamine are dependent on the level of $[Na^+]_i$. Therefore, the effect of hormones that regulate the rate of sodium translocation across the proximal tubule epithelial cells, and thereby sodium excretion, may be modulated by the level of $[Na^+]_i$. The experiments described in this study relied on exogenously added 8-OH-DPAT, dopamine and PMA. Hence, the modulating effect on the Na^+,K^+-ATPase activity by these reagents is likely the result of a permissive effect of elevated $[Na^+]_i$ on signalling molecule(s) that are downstream of the hormone receptors. Thus, the level of $[Na^+]_i$ may be a major determinant to balance the activity of hormones that have antagonistic effects on the regulation of proximal tubule sodium reabsorption.

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