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Short communication

Activation of dopamine D_1-like receptor causes phosphorylation of α_1-subunit of Na^+,K^+-ATPase in rat renal proximal tubules

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Abstract

Dopamine causes inhibition of Na^+,K^+-ATPase activity via activation of dopamine D_1-like receptors. It is the phosphorylation of Serine[18] of the α_1-subunit of Na^+,K^+-ATPase which results in the inhibition of the enzyme activity; however, such a phosphorylation by dopamine D_1-like receptor agonist has not been demonstrated in the proximal tubules. We show here by immunoprecipitation and detection with phosphoserine antibody that SKF 38393, a dopamine D_1-like receptor agonist, causes phosphorylation of the α_1-subunit of Na^+,K^+-ATPase. The effect of (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride, SKF 38393, is blocked by *R*(+)-7-choro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride, SCH 23390, a dopamine D_1-like receptor antagonist, and staurosporin, a protein kinase C inhibitor. The phosphorylation is also increased by phorbol 12–13 dibutyrate ester. However, Rp-cAMP triethylamine, an inhibitor of protein kinase A, does not affect the SKF 38393-mediated phosphorylation of Na^+,K^+-ATPase. Therefore, these results provide the evidence that dopamine D_1-like receptor activation causes phosphorylation of the α_1-subunit of Na^+,K^+-ATPase in renal proximal tubules via protein kinase C pathway.

Keywords: SKF 38393; Protein kinase C; Protein kinase A; Na^+,K^+-ATPase; Dopamine D_1-like receptors; Dopamine

1. Introduction

Dopamine promotes renal sodium excretion, and plays a role in maintaining the sodium homeostasis (Lokhandwala and Amenta, 1991; Hussain and Lokhandwala, 1998). Dopamine is synthesized within the renal proximal tubular cells, and is released into the tubular lumen and on the basolateral side where it acts in an autocrine fashion by activating dopamine D_1-like receptors (Baines and Chan, 1980; Zimlichman et al., 1988; Chen and Lokhandwala, 1993; Satoh et al., 1993). The inhibitory effect of dopamine on tubular sodium reabsorption results from the activation of dopamine D_1-like receptors located on proximal tubules and subsequent inhibition of the Na^+,H^+-exchanger and Na^+,K^+-ATPase (Hussain and Lokhandwala, 1998).

Numerous studies have shown that hormonal regulation of Na^+,K^+-ATPase activity can be accomplished by phosphorylation/dephosphorylation of its α_1-subunit catalyzed by protein kinase and phosphatase, respectively (Ribero and Mandel, 1992; Aperia et al., 1996). Phosphorylation sites on the α_1-subunit of Na^+,K^+-ATPase for protein kinase A (Beguin et al., 1994; Fisone et al., 1994) and protein kinase C (Beguin et al., 1994; Logvinenko et al., 1996) have been identified and their functional significance has been verified by site-directed mutagenesis (Fisone et al., 1994; Pedemonte et al., 1997). The inhibition of Na^+,K^+-ATPase activity via phosphorylation results from either the intrinsic change in Na^+,K^+-ATPase, which include a change in conformational equilibrium (Logvinenko et al., 1996) and alterations in the affinity for Na^+ (Fisone et al., 1994) or K^+ (Logvinenko et al., 1996), or internalization of Na^+,K^+-ATPase molecule from the plasma membrane (Chibalin et al., 1998, 1999). Dopamine induced phosphorylation of α_1-subunit and its association with inhibition of the Na^+,K^+-ATPase activity via a protein kinase C-dependent pathway has been reported in renal proximal tubular cells and epithelial cells (Opposum kidney cell line) transfected with rat α_1-subunit of Na^+,K^+-ATPase (Chibalin et al., 1998, 1999). Moreover, inhibition of the Na^+,K^+-ATPase by dopamine in single permeabilized renal proximal tubule has also been linked

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to simultaneous activation of both dopamine D_1-like and D_2-like receptors (Bertorello and Aperia, 1990).

Although specific activation of dopamine D_1-like receptors by preferential agonists such as fenoldopam and (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride, SKF 38393, is reported to inhibit Na^+,K^+-ATPase activity (Felder et al., 1989, Yao et al., 1998), the effect of dopamine D_1-like receptor activation alone in the phosphorylation process itself has not yet been reported. Therefore, the present study was carried out to determine the effect of SKF 38393 on the phosphorylation of the α_1-subunit of Na^+,K^+-ATPase in the rat proximal tubule and to identify the role of protein kinases (protein kinase A and C) in this phenomenon.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats of 150–200 g (Harlan Sprague–Dawley, Indianapolis, IN, USA) were anesthetized with pentobarbital (50 mg/kg, i.p.), a midline incision was made, kidneys were excised and kept in ice cold oxygenated incubation medium containing (mM): $CaCl_2$ 1.5, NaCl 110, KCl 5.4, KH_2PO_4 1, $MgSO_4$ 1, $NaHCO_3$ 25, D-glucose 25, HEPES 2, (pH 7.6). Transverse sections (2–3 mm thick) of the kidneys were cut with a razor blade and kept in fresh cold medium. Superficial cortical tissue slices (400–500 μm) were dissected out carefully, and used immediately for drug treatments.

2.2. Drug treatment

Renal cortical tissue slices (rich in proximal tubular cells) were placed in fresh incubation medium and prewarmed at 30°C for 5 min while oxygenating. The tissue slices were then washed once and preincubated with 1 μM okadaic acid for 5 min in incubation medium. (±)-1-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride, SKF 38393 (10 μM), treatment was carried out in the absence and presence of either *R*(+)-7-choro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride (SCH 23390, 10 μM), staurosporin (0.1 μM) or Rp-cAMP triethylamine (100 μM) for 20 min. Phorbol 12–13 dibutyrate (PDB, 1 μM) treatment was conducted in the absence or presence of staurosporin (0.1 μM) for 20 min. *R*(+)-7-choro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride, SCH 23390, staurosporin and Rp-cAMP triethylamine were added 5 min prior the addition of either SKF 38393 or PDB. All the incubations were performed at 30°C while oxygenating. After the drug treatment, medium was aspirated, cortical tissue slices were washed once with cold incubation medium and quickly frozen on dry ice–ethanol mixture.

2.3. Na^+,K^+-ATPase immunoprecipitation

Cortical tissue slices were thawed on ice and homogenized in immunoprecipitation buffer containing (mM): Tris–HCl 20, NaCl 150, NaF 10, $Na_2P_4O_7$ 10, ethylenediaminetetraacetic acid (EDTA) 2, ethyleneglycoltetraacetic acid 2, 1% triton X-100, 0.1% sodium dodecyl sulfate (SDS), (pH 8.0), protease inhibitor cocktail and 0.1 mM phenylmethanesulfonyl fluoride. Homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant was precleared with Protein A/G-agarose for 30 min. Precleared supernatant (2 mg/ml) was incubated with mouse monoclonal α_1-subunit of Na^+,K^+-ATPase antibody. Antigen (α_1-subunit of Na^+,K^+-ATPase)-antibody complex was precipitated using Protein A/G-agarose. The complex was washed once with immunoprecipitation buffer followed by three washes with buffer containing (mM): Tris–HCl 20, NaCl 150, EDTA 5, 0.1% triton X-100, 0.1% SDS, and once with 50 mM Tris–HCl, (pH 8.0). Finally, the antigen–antibody complex was dissociated in 50 μl of 2× Laemmeli buffer (125 mM Tris–HCl, 4% SDS, 5% β-mercaptoethanol, 20% glycerol) at 37°C for 1 h. All the steps of immunoprecipitation were carried out at 4°C unless otherwise specified.

2.4. Na^+,K^+-ATPase dephosphorylation

Kidney slices were treated in the absence or presence of SKF 38393 (10 μM for 20 min) and Ag–Ab complex (Na^+,K^+-ATPase bound to Protein A/G-agarose) was obtained in the same manner as described above. The complex was washed once with immunoprecipitation buffer and 4× with assay buffer containing (mM): Tris–HCl 50, EDTA 0.1, dithiothretol 5, $MnCl_2$ 2, 0.01% polyhydroxyethylene ether (pH 7.5). Na^+,K^+-ATPase bound to Protein A/G-agarose was incubated in the absence or presence of 400 units of protein phosphatase (CalbioChem) in 40 μl of assay buffer for 30 min at 30°C. The incubation was terminated by adding 20 μl of Laemmeli buffer and warming at 37°C for 1 h.

2.5. Na^+,K^+-ATPse immunoprecipitation from Opposum kidney (OK) cells

OK cells transfected with wild type and double mutated (Ser^{11A} and Ser^{18A}) rat α_1 cDNA of Na^+,K^+-ATPase were grown in Dulbecco's modified Eagle's medium (for transfection see Efendiev et al., 2000). The cells (80% confluent) were washed with PBS, incubated with 1 μM phorbol 12-myristate 13-acetate (PMA) for 10 min at room temperature. The cells were washed and taken in ice cold immunoprecipitation buffer and immediately frozen in liquid nitrogen. After thawing, cells were lysed by sonication and Na^+,K^+-ATPase was immunoprecipitated in the same manner as detailed above.

2.6. Na^+,K^+-ATPase phosphorylation

The immunoprecipitated samples were resolved by SDS polyacrylamide gel (8%) electrophoresis. The resolved proteins were electrophoretically transblotted on membrane (Immobilon-P). The membrane was blocked with 5% nonfat dry milk followed by incubation with mouse monoclonal phosphoserine antibody for 1 h. Horseradish peroxidase conjugated goat anti-mouse second antibody incubation was performed for 1 h at room temperature. The membrane was treated with enhanced chemiluminescent reagent and the bands were visualized on X-ray film. The bands were quantitated by densitometry analysis using Quantity One software program.

2.7. Na^+,K^+-ATPase protein

The protein of Na^+,K^+-ATPase in the immunoprecipitated samples was detected by Western blotting following the same procedure described above except that mouse monoclonal α_1-subunit of Na^+,K^+-ATPase antibody was used as primary antibody.

2.8. Protein measurement

Protein in cortical homogenate and in OK cell lysate were measured using bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and bovine serum albumin as standards.

2.9. Statistics

Results are presented as mean ± S.E.M. Statistical significance was measured using Student's *t*-test (unpaired) and $P < 0.05$ was considered significant.

2.10. Materials

(±)-1-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF 38393), *R*(+)-7-Choro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride (SCH 23390), Rp-cAMP triethylamine, Staurosporin and PDB were purchased from Research Biochemicals International (Natick, MA). Okadaic acid, protein phosphatase enzyme and phosphoserine antibody were purchased from Calbiochem (San Diego, CA). Monoclonal α_1-subunit Na^+,K^+-ATPase antibody was purchased from Research Diagnostics (Flanders, NJ). Protein A/G-agarose was bought from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals for various buffers were of the highest purity available and purchased either from Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

3. Results

The monoclonal antibody against α_1-subunit of Na^+,K^+-ATPase immunoprecipitated approximately 101 kDa protein, which showed phosphorylation upon detection with the phosphoserine antibody. In order to examine the specificity of the phosphoserine antibody, we used OK cells transfected with wild type or double mutated (Ser^{11A} and Ser^{18A}) α_1 cDNA of the Na^+,K^+-ATPase. We found that the phosphorylation of Na^+,K^+-ATPase in the presence of phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, in the mutated α_1-subunit transfected cells was lesser than in the cells transfected with wild type α_1-subunit (Fig. 1A). In another set of experiments carried out in the cortical samples, we found that dopamine D_1-like receptor agonist, SKF38393-mediated

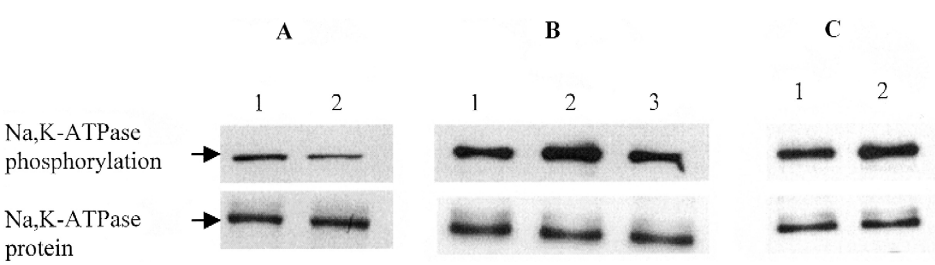

Fig. 1. (A) Wild type and double (Ser^{11A} and Ser^{18A}) mutated α_1-subunit cDNA of Na^+,K^+-ATPase transfected OK cells were treated with 1 μM phorbol 12-myristate 13-acetate (PMA) for 10 min, Na^+,K^+-ATPase immunoprecipitated, electrophoresed (5 μl), transblotted and detected for phosphorylation (upper panel blot) and Na^+,K^+-ATPase protein (lower panel blot) using phosphoserine and Na^+,K^+-ATPase antibodies, respectively (see Materials and methods). PMA induced phosphorylation of Na^+,K^+-ATPase in wild type (lane 1) but not in double (Ser^{11A} and Ser^{18A}) mutated (lane 2) α_1-subunit cDNA transfected cells. (B) Kidney slices were first treated with or without SKF 38393 (10 μM) for 20 min, Na^+,K^+-ATPase immunoprecipitated, and then treated in the absence or presence of phosphatase enzyme (400 units), solubilized in Laemmeli buffer, electrophoresed (10 μl), transblotted and detected for Na^+,K^+-ATPase phosphorylation (upper panel blot) and Na^+,K^+-ATPase protein (lower panel blot). Basal phosphorylation of Na^+,K^+-ATPase (lane 1), SKF 38393-induced phosphorylation (lane 2) was dephosphorylated by phosphatase enzyme (lane 3). (C) Kidney slices were treated in the absence (lane 1) or presence (lane 2) of 1 μM okadaic acid for 20 min, Na^+,K^+-ATPase immunoprecipitated, electrophoresed (10 μl), transblotted and detected for Na^+,K^+-ATPase phosphorylation (upper panel blot) and Na^+,K^+-ATPase protein (lower panel blot). Okadaic acid increased the basal phosphorylation of Na^+,K^+-ATPase.

phosphorylation was reduced by treating the samples with exogenous phosphatase (Fig. 1B), whereas the basal phosphorylation was increased by adding phosphatase inhibitor, okadaic acid to the incubation sample (Fig. 1C). However, we did not see a complete ablation of phosphoserine signal by the phosphatase, which could be due to low enzyme units used in the study. All together, these results suggest that the antibody used in this study was specific for phoshoserine and was able to detect changes in the phosphorylation of Na^+,K^+-ATPase induced by various agents.

The effect of SKF 38393, a dopamine D_1-like receptor agonist and PDB, a direct activator of protein kinase C on Na^+,K^+-ATPase phosphorylation was measured as the ratio of the density of phosphorylated Na^+,K^+-ATPase (Fig. 2, upper panel) to the total Na^+,K^+-ATPase protein (Fig. 2, lower panel, lower band) and plotted in Fig. 2D. SKF 38393 enhanced the level of phosphorylation of Na^+,K^+-ATPase compared to control (percent of basal: SKF 38393, 130.85 ± 1.13; Fig. 2A and D). SKF 38393-induced phosphorylation of Na^+,K^+-ATPase was attenuated by a specific dopamine D_1-like receptor antagonist, SCH 23390 (percent of basal: SKF 38393 + SCH 23390, 109.60 ± 1.31; Fig. 2A and D). It should be noted that we observed a doublet of the Na^+,K^+-ATPase in immunoprecipitate samples. Of the two protein bands, only 101 kDa band was detected with another antibody (called α5) raised against α_1-subunit of Na^+,K^+-ATPase (Koster et al., 1995, data not shown) confirming that the 101 kDa protein represented Na^+,K^+-ATPase.

In order to identify the role of protein kinases in this process, cortical tissue slices were treated with SKF 38393, a dopamine D_1-like receptor agonist in the presence of either staurosporin, a protein kinase C inhibitor or Rp-cAMP triethylamine, a protein kinase A inhibitor. SKF 38393-induced phosphorylation of Na^+,K^+-ATPase was blocked by staurosporin (percent of basal: SKF 38393 + staurosporin, 103.40 ± 0.53; Fig. 2A and D). Rp-cAMP triethylamine did not affect SKF 38393-induced phosphorylation of Na^+,K^+-ATPase (percent of basal: SKF 38393 + Rp-cAMP triethylamine, 130.00 ± 1.24; Fig. 2B and D). Phorbol 12–13 dibutyrate ester, a direct activator of protein kinase C alone increased the phosphorylation of

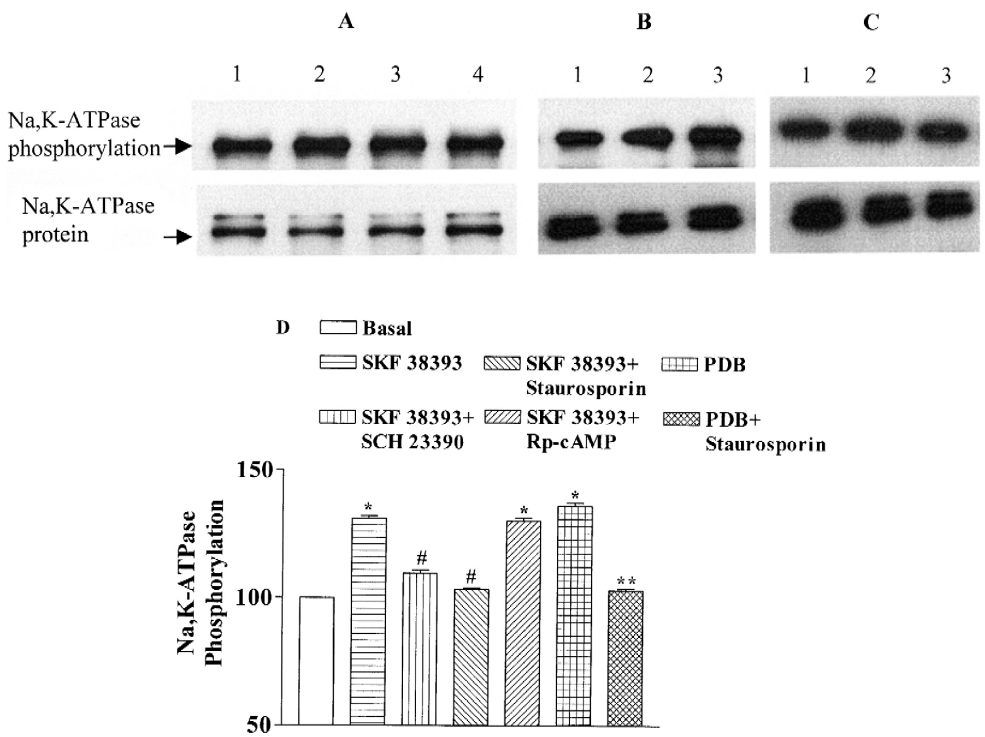

Fig. 2. (A) Kidney slices were treated in the absence or presence of either SKF 38393 (10 μM) or PDB (1 μM) alone for 20 min. SCH 23390 (10 μM) and Rp-cAMP triethylamine (100 μM) treatment was done in the presence of SKF 38393 (10 μM) for 20 min. Staurosporin (0.1 μM) treatment was performed either with SKF 38393 (10 μM) or PDB (1 μM) for 20 min. Na^+,K^+-ATPase was immunoprecipitated, electrophoresed (10 μl), transblotted and detected for Na^+,K^+-ATPase phosphorylation (upper panel, representative blot) and Na^+,K^+-ATPase protein (lower panel, representative blot). (A) Basal phosphorylation of Na^+,K^+-ATPase (lane 1), SKF 38393-induced phophorylation of Na^+,K^+-ATPase (lane 2) was blocked by SCH 23390 (lane 3) and staurosporin (lane 4). (B) Basal phosphorylation of Na^+,K^+-ATPase (lane 1), SKF 38393-mediated phosphorylation of Na^+,K^+-ATPase (lane 2) was unaffected by Rp-cAMP triethylamine (lane 3). (C) Basal phosphorylation of Na^+,K^+-ATPase (lane 1), PDB-induced phosphorylation of Na^+,K^+-ATPase (lane 2) were attenuated by staurosporin (lane 3). (D) Bar diagram presentation of the ratio of the densities between phosphorylated Na^+,K^+-ATPase (upper panel) and Na^+,K^+-ATPase protein (lower panel). Values are expressed as percent of basal (represented as 100) and are means ± S.E.M. of 3–7 experiments: SKF 38393 alone (130.85 ± 1.13), SKF 38393 together with either SCH 23390 (109.60 ± 1.31), staurosporin (103.40 ± 0.53) or Rp-cAMP triethylamine (130.00 ± 1.24), PDB alone (136.00 ± 1.27) and PDB together with staurosporin (103.00 ± 0.70). * —Compared with basal; # —compared with SKF 38393 alone; ** —compared with PDB alone ($P < 0.05$, Student's *t*-test).

Na^+,K^+-ATPase, which was abolished by staurosporin, a specific inhibitor of protein kinase C (percent of basal: PDB, 136.00 ± 1.2; PDB + staurosporin, 103.00 ± 0.70; Fig. 2C and D).

4. Discussion

The present study demonstrated that SKF 38393 increased phosphorylation of Na^+,K^+-ATPase in the proximal tubules of rat kidney which was antagonized by SCH 23390 and staurosporin. These findings suggest that the activation of dopamine D_1-like receptors via protein kinase C causes phosphorylation of Na^+,K^+-ATPase.

Dopamine does not distinguish between dopamine D_1-like and D_2-like receptors and acts on both the subtypes of the receptors. Therefore, dopamine induced phosphorylation and inhibition of Na^+,K^+-ATPase may be due to the involvement of both dopamine D_1-like and D_2-like receptors. In fact, Bertorello and Aperia (1990) have shown that inhibition of Na^+,K^+-ATPase activity in proximal tubules by dopamine requires the simultaneous activation of both dopamine D_1-like and D_2-like receptors. Phosphorylation of the α_1-subunit of Na^+,K^+-ATPase is suggested to induce intrinsic changes in the enzyme molecule and subsequent inhibition of Na^+,K^+-ATPase activity (Fisone et al., 1994; Logvinenko et al., 1996). However, Chibalin et al. (1998, 1999) suggest that phosphorylation induced inhibition of Na^+,K^+-ATPase activity in proximal tubule is a result of internalization of the enzyme molecule from the plasma membrane. Nevertheless, it has been established that the first step in the process of inhibition of the Na^+,K^+-ATPase activity is phosphorylation of its α_1-subunit (Beguin et al., 1994; Fisone et al., 1994). Our results show that the specific activation of the dopamine D_1-like receptors also leads to phosphorylation of α_1-subunit of the Na^+,K^+-ATPase.

We discovered that in addition to SKF 38393, PDB, a direct activator of protein kinase C, also induced phosphorylation of Na^+,K^+-ATPase in proximal tubules and the effect of both SKF 38393 and PDB was abolished by staurosporin, a protein kinase C inhibitor. The observation that the effect of SKF 38393 on phosphorylation of Na^+,K^+-ATPase was abolished by staurosporin but not by Rp-cAMP triethylamine suggests the involvement of protein kinase C but not protein kinase A pathway in this phenomenon. While we did not measure the activity of Na^+,K^+-ATPase in response to SKF 38393 in this study, earlier reports from our (Kansra et al., 1995; Chen and Lokhandwala, 1993) and other (Felder et al., 1989; Yao et al., 1998) laboratories have shown that dopamine and dopamine D_1-like receptor agonists cause inhibition of the Na^+,K^+-ATPase activity in proximal tubules via protein kinase C-dependent pathway. In the more distal portions of the nephron, protein kinase A is reported to play a role in the regulation of Na^+,K^+-ATPase activity (Kiroytcheva et al., 1999).

In summary, our results show that the activation of dopamine D_1-like receptors by SKF 38393 leads to the phosphorylation of α_1-subunit at serine residues of Na^+,K^+-ATPase in the proximal tubules of rat kidney, via a protein kinase C-dependent pathway. Such a phosphorylation might be a factor in dopamine D_1-like receptors mediated inhibition of Na^+,K^+-ATPase reported earlier in the rat proximal tubules.

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