Hyperphosphorylation of Na-Pump Contributes to Defective Renal Dopamine Response in Old Rats

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Abstract. Dopamine D_1-like receptor activation causes phosphorylation and inhibition of Na,K-ATPase (Na-pump) activity in the proximal tubules, which is associated with an increase in sodium excretion. It has been shown that dopamine and SKF 38393, a D_1-like receptor agonist, caused inhibition of Na,K-ATPase activity in the proximal tubules of adult (6 mo) but not of old (24 mo) Fischer 344 rats. The present study demonstrated that SKF 38393 and PDBu, a phorbol ester and protein kinase C (PKC) activator, increased phosphorylation of the α_1-subunit of Na,K-ATPase in adult but not in old rats. In adult rats, SKF 38393-mediated phosphorylation was antagonized by SCH 23390, a D_1-like receptor antagonist. Similarly, Na,K-ATPase activity was inhibited by SKF 38393 and PDBu in adult but not in old rats. The basal activity of Na,K-ATPase was decreased and the basal phosphorylation state of the enzyme was increased in old compared with adult rats. Basal activity of PKC was higher in old compared with adult rats, and SKF 38393 and PDBu stimulated PKC activity in adult but not in old rats. The conclusion is that the failure of D_1-like receptor agonist and phorbol ester to stimulate PKC and inhibit Na,K-ATPase activity in old rats is due, at least in part, to the higher basal PKC activity and Na,K-ATPase phosphorylation in old compared with adult rats.

Dopamine promotes natriuresis and diuresis by activation of D_1-like dopamine receptors located in different regions of the nephron (1–3). Dopamine D_1-like receptor activation leads to its coupling with $G_{q/11}$ protein and results in phosphorylation and inhibition of Na,K-ATPase activity in the proximal tubules (PT) via the phospholipase C-linked protein kinase C (PKC) pathway (4–8). In addition, coupling of D_1-like receptor and G_s protein triggers a cAMP-linked protein kinase A signaling cascade that phosphorylates and inhibits the activity of Na,H exchanger (6,9,10). Both of these phenomena contribute to the D_1-like receptor-mediated inhibition of sodium reabsorption.

It is reported that the natriuretic response to dopamine and SKF 38393, a D_1-like receptor agonist, is reduced in spontaneously hypertensive rats (SHR) compared with Wistar Kyoto (WKY) rats (11,12). Such a blunted response to D_1-like receptor stimulation in SHR is reportedly due to a defective D_1-like receptor coupled signal transduction pathway, which causes a reduced inhibition of Na,K-ATPase and Na,H exchanger in SHR (13–15). A similar defect in D_1-like receptor signaling and function was also found in the PT of humans with essential hypertension (16). Furthermore, this study (16) suggested that the defective function of D_1-like receptor may be due to an increased level of ligand-independent phosphorylation of the receptor, which leads to its uncoupling from the G proteins. In addition to such a defect at the level of D_1-like receptor, it was found that there was a D_1-like agonist-induced differential regulation of PKC isoforms (PKC-δ and PKC-ζ) in SHR and WKY rats (17), which contributed to the failure of dopamine to stimulate PKC (18) and inhibit Na,K-ATPase activity (13) in the PT of SHR. The basal PKC activity (18) and PDBu-mediated inhibition of Na,K-ATPase (13) was not different in SHR compared with WKY rats.

The normal aging process leads to structural changes in the kidney, which have been associated with changes in renal function such as decline in renal blood flow and GFR (19,20). Renal deficiency in L-dopa uptake and its conversion to dopamine (21) and a reduced dopamine receptor number and its defective coupling with G proteins have been reported in the kidney of old rats (22). Recently, we reported that dopamine failed to inhibit Na,K-ATPase activity in old rats, in part due to a decreased number of D_1-like receptor-binding sites and defective D_1-like receptor-G protein coupling in the PT of these animals (22). In addition to a defect at the level of D_1-like receptor, it is likely that there may exist alterations in other components of the signal transduction pathway, such as PKC activity and the phosphorylation state of Na,K-ATPase itself, which would contribute to the failure of dopamine to inhibit Na,K-ATPase activity in old rats. Therefore, the present study was designed to examine the potential alterations in receptor-mediated (using D_1-like agonist SKF 38393) and non–receptor-mediated (using PKC activator PDBu) activation in PKC activity and phosphorylation of the α_1-subunit of Na,K-ATPase in adult and old rats. Our results show that there is an increase in the basal PKC activity and subsequent phosphorylation of the Na,K-ATPase in old rats under resting state. This

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abnormality beyond the level of D_1-like receptor may also contribute to the failure of receptor- and non–receptor-mediated phosphorylation and subsequent inhibition of Na,K-ATPase in old rats.

Materials and Methods

Animals

Male Fischer 344 rats of 6 (adult) and 24 (old) months of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The rats were housed in plastic cages in an animal care facility and fed normal rat chow and water *ad libitum*.

Isolation and Enrichment of PT

Rat kidneys were used to prepare and enrich proximal tubular suspension according to a routinely used procedure in our laboratory and described elsewhere (13).

PKC Assay

Renal PT were incubated with SKF 38393 (1 nM to 1 μM) or PDBu (1 nM to 1 μM) for 20 min at 30°C. PKC activity was measured using a fluorescence peptide substrate (PLSRTLSVAAK) as described in the PKC assay kit manufacturer's manual (Promega, Madison, WI) and as we have described earlier (8). The fluorescence peptide phosphorylated by the activation of PKC was resolved on a 0.8% agarose gel. The gel was photographed under ultraviolet light, and the bands were densitometrically quantified.

Na,K-ATPase Assay

The PT were treated with PDBu (1 nM to 1 μM) or SKF 38393 (1 nM to 1 μM) for 20 min at 37°C. The incubation was terminated by rapid freezing on dry ice-acetone and thawing. After thawing, the samples were used for Na,K-ATPase assay using end phosphate hydrolysis of ATP and colorimetric measurement as routinely used in our laboratory and described earlier (13). Ouabain-sensitive ATPase (Na,K-ATPase) was calculated as the difference between total ATPase activity and ATPase activity in the presence of 1 mM ouabain. The sodium concentration used in this set of Na,K-ATPase assay was 75 mM, which is a saturating (Vmax) concentration (23).

Basal activity of Na,K-ATPase was also measured in the membranes prepared from the outer cortex of the kidneys at low sodium (5 mM) as well as saturating sodium (75 mM) concentration (23). Low sodium (5 mM) in the reaction mixture was compensated with Tris buffer to maintain osmolality. Other details of the assay are similar as described above.

Immunoprecipitation of Na,K-ATPase

Transverse sections of the kidney were obtained with the help of a razor blade. Under magnifying glass, thin superficial cortical tissue slices (approximately 400 to 500 μm, rich in PT) were carefully dissected using a razor blade. The tissue slices were placed in ice-cold oxygenated buffer containing 1.5 mM $CaCl_2$, 110 mM NaCl, 5.4 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 25 mM D-glucose, and 2 mM HEPES (pH 7.6). The tissue slices were preincubated with 1 μM okadaic acid and treated with SKF 38393 (10 μM) or PDBu (1 μM) for 20 min at 30°C while oxygenating. SCH 23390 (10 μM, a D_1-like receptor antagonist) was added 5 min before the addition of SKF 38393. The incubation was terminated by quickly freezing the tissues on dry ice-acetone mixture. The tissues were homogenized in immunoprecipitation buffer containing 20 mM Tris-HCl, 150 mM NaCl, 10 mM NaF, 10 mM $Na_2P_4O_7$, 2 mM ethylenediaminetetraacetate, 2 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1% triton X-100, 0.1% sodium dodecyl sulfate (SDS; pH 8.0), protease inhibitor cocktail, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenate was centrifuged at 8000 $\times$ g for 15 min, and the supernatant was precleared with Protein A/G Plus agarose for 30 min. An aliquot of precleared supernatant (2 mg of protein/ml) was incubated with mouse monoclonal antibody of the α_1-subunit of Na,K-ATPase (24). Antigen (α_1-subunit of Na,K-ATPase)-antibody complex was precipitated using ProA/G-agarose. The complex was washed once with immunoprecipitation buffer followed by three washes with buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetate, 0.1% triton X-100, 0.1% SDS, and once with 50 mM Tris-HCl (pH 8.0). All of the steps of immunoprecipitation were carried out at 4°C. Finally, the antigen-antibody complex was dissociated with 50 μl of 2X Laemmeli buffer (125 mM Tris-HCl, 4% SDS, 5% β-mercaptoethanol, 20% glycerol) by incubating at 37°C for 1 h.

Measurement of Phosphorylated α_1-Subunit of Na,K-ATPase

The immunoprecipitated sample (10 μl) was resolved by SDS/polyacrylamide gel (8%) electrophoresis. The resolved proteins were electrophoretically transblotted on membrane (Immobilon-P, Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat dry milk followed by incubation with mouse monoclonal phosphoserine antibody for 60 min. Horseradish peroxidase conjugated goat anti-mouse second antibody incubation was performed for 60 min at room temperature. The membrane was treated with enhanced chemiluminescence reagent, and the bands were visualized on x-ray film. The bands were quantitated by densitometry analysis using the Quantity One software program (pdi, Inc., Huntington Station, NY).

Measurement of Total α_1-subunit of Na,K-ATPase

The total (phosphorylated and nonphosphorylated) contents of the Na,K-ATPase α_1-subunit in the immunoprecipitated samples and isolated membranes were detected by Western blotting following the same procedure described above except that the mouse monoclonal α_1-subunit of Na,K-ATPase antibody was used as the primary antibody.

Measurement of Protein

Protein in cortical homogenates and in the membranes was measured using BCA protein assay kit (Pierce, Rockford, IL) and bovine serum albumin as standards.

Materials

SKF 38393, SCH 23390 and PDBu were purchased from Research Biochemical International (Natick, MA). Okadaic acid and phosphoserine antibody were purchased from Calbiochem (San Diego, CA). Monoclonal α_1-subunit Na,K-ATPase antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ). Protein A/G Plus agarose was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All of the other chemicals of the highest purity available for various buffers were purchased either from Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific Co. (Fair Lawn, NJ).

Statistical Analyses

Results are presented as mean $\pm$ SEM. Statistical significance was measured using t test (within the group) and ANOVA (between the groups). $P < 0.05$ was considered significant.

Results

Effect of SKF 38393 or PDBu on Na,K-ATPase Activity

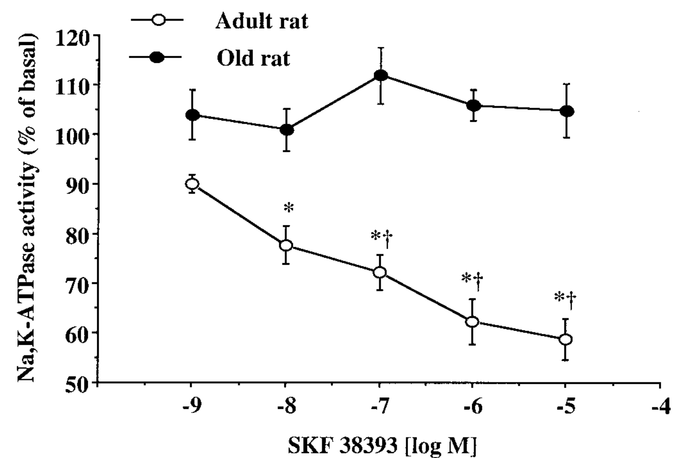
SKF 38393 (1 nM to 1 μM) and PDBu (1 nM to 1 μM) elicited a concentration-dependent inhibition of Na,K-ATPase activity in adult but not in old rats (Figure 1). The basal activity of Na,K-ATPase (nmol Pi/mg protein per min) in the presence of 75 mM sodium was lower in the PT of old (109 ± 5) compared with adult (136 ± 9) rats.

In the cortical membranes, the basal activity of Na,K-ATPase in old rats was lower by 42% at a lower sodium concentration (5 mM) and by 25% at saturating sodium concentration (75 mM) compared with adult rats (adult *versus* old: at 5 mM sodium, 307 ± 25 *versus* 178 ± 19 nmol Pi/mg protein per min; at 75 mM sodium, 746 ± 66 *versus* 555 ± 14 nmol Pi/mg protein per min; Figure 2A).

Effect of SKF 38393 or PDBu on Na,K-ATPase Phosphorylation

Mouse monoclonal α_1-subunit of Na,K-ATPase antibody recognized two protein bands in the immunoprecipitate. The lower band (approximately 101 kD) of the doublet showed phosphorylation upon detection with phosphoserine antibody. To confirm that 101 kD protein is the real α_1-subunit, we performed Western blot analysis of the immunoprecipitate with another monoclonal α_1-subunit antibody, 5α, kindly provided by Dr. Carlos Pedemonte (University of Houston, Houston, TX). This antibody recognized only the lower band of the doublet, confirming it as α_1-subunit (data not shown). The antibody 5α has been shown to recognize only one protein band in various tissues, including in the kidney (25). Phosphorylation of Na,K-ATPase was calculated as the ratio of arbitrary density unit of phosphorylated Na,K-ATPase to the total Na,K-ATPase protein (lower band, approximately 101 kD). The basal phosphorylation of Na,K-ATPase was significantly higher in old compared with adult rats (adult *versus* old: 1.47 ± 0.12 *versus* 2.29 ± 0.39; Figure 2B). However, basal abundance of Na,K-ATPase protein was the same in the cortical membranes of both adult and old rats (adult *versus* old: 1.42 ± 0.02 *versus* 1.33 ± 0.02 arbitrary units; Figure 2C). As shown in Figure 3, SKF 38393 (10 μM) or PDBu (1 μM) increased phosphorylation (by 33 and 43%, respectively) of α_1-subunit of Na,K-ATPase in the cortex slices of adult rats but not in old rats. The stimulatory effect of SKF 38393 (10 μM) on phosphorylation in adult rats was attenuated by SCH 23390 (10 μM), a specific D_1-like receptor antagonist (Figure 3).

To confirm the linearity of protein immunodetection, we loaded 5, 10, and 15 μl of immunoprecipitated samples from 6-mo old rats. Western blot analysis for both phosphorylated and total α1 protein was performed. The densitometric values (for 5, 10, and 15 μl, respectively) were as follow: 0.69, 1.41, and 2.01 for phosphorylated and 0.55, 1.07, and 1.57 for total α_1 protein. The ratio of phosphorylated to total α_1 protein (1.25, 1.31, and 1.26) did not change with the change in sample quantity. The data suggest that the amount used (10 μl) in the rest of the experiments was in linear range of immunodetection of the proteins in our samples.



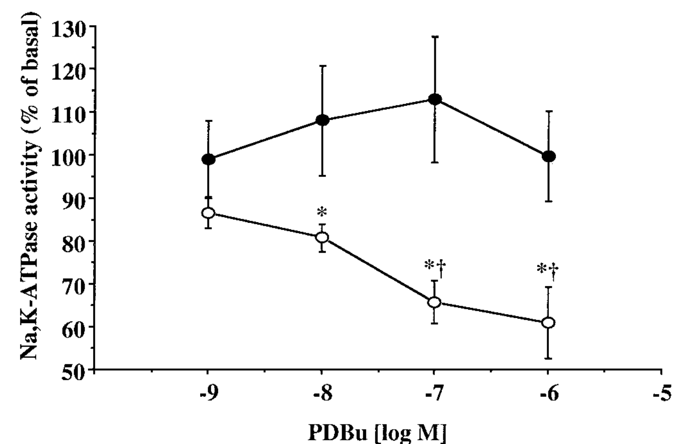


Figure 1. Effect of SKF 38393 or PDBu on Na,K-ATPase activity in the proximal tubules (PT) of adult and old rats. The basal activity of Na,K-ATPase in adult and old rats was 136 ± 9 and 109 ± 5 nmol Pi/mg protein per min, respectively. The values represent mean ± SEM of four to six experiments performed in triplicate. *, significant difference within the group from basal (one-way ANOVA, $P < 0.05$); †, significant difference between groups (*t* test, $P < 0.05$).

Effect of SKF 38393 or PDBu on PKC Activity

SKF 38393 (1 nM to 1 μM) stimulated PKC activity in the PT of adult rats, whereas D_1-like receptor activation failed to stimulate the enzyme activity in the old rats (Figure 4A). Similarly, PDBu (1 nM to 1 μM), a phorbol ester and direct activator of PKC, stimulated PKC activity in the adult but not in the old rats (Figure 4B). The basal PKC activity (peptide phosphorylated ng/μg protein per 20 min) was significantly higher in old (15.2 ± 2.6) compared with adult (7.3 ± 1.1) rats.

Discussion

We previously reported that dopamine and SKF 38393, a D_1-like receptor agonist, failed to inhibit Na,K-ATPase and Na,H exchanger in old rats, and the natriuretic and diuretic response to these agonists was also reduced in old compared with adult rats (22,26). Some of the factors that contribute to such a defective dopaminergic response in old rats include a decrease in D_1-like receptor-binding sites in both basolateral

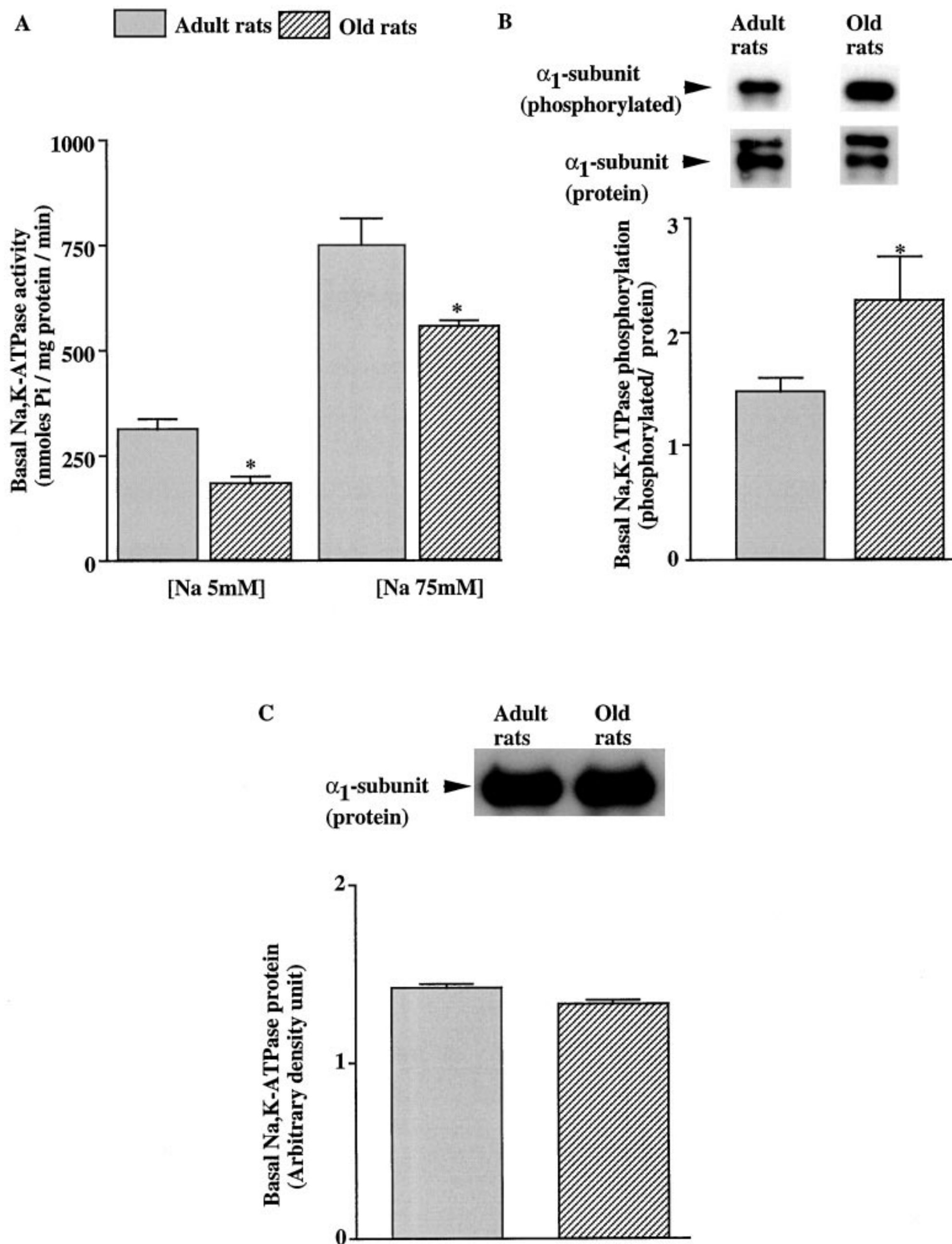


Figure 2. Basal activity, phosphorylation, and protein content of Na,K-ATPase in adult and old rats. (A) Basal activity at low (Na 5 mM) and high (Na 75 mM) sodium concentration in the membranes (adult *versus* old: at Na 5 mM 307 ± 25 *versus* 178 ± 19; at Na 75 mM, 746 ± 66 *versus* 555 ± 14 nmol Pi/mg protein per min; $n = 3$). (B) Basal phosphorylation was measured as the ratio of arbitrary density unit of phosphorylated Na,K-ATPase and the total protein content of the enzyme in the immunoprecipitate from the cortex slices. Upper panel: representative blot; lower panel: mean ± SEM; $n = 4$ (adult *versus* old, 1.47 ± 0.12 *versus* 2.29 ± 0.39). (C) Basal protein content of Na,K-ATPase in the membranes. Upper panel: representative blot; lower panel: mean ± SEM; $n = 3$ (adult *versus* old: 1.42 ± 0.02 *versus* 1.33 ± 0.02 arbitrary density unit). *, significant difference from adult (t test, $P < 0.05$).

and brush border membranes and a defective coupling of D_1-like receptors to G_s and $G_{q/11}$ proteins (22,26).

The present study was performed to determine whether there existed a defect at the level of Na,K-ATPase that may also have contributed to the failure of dopamine to inhibit the enzyme activity. In support of a defect at the level of Na,K-ATPase, we found that in addition to SKF 38393, a direct activator of PKC, namely PDBu also failed to inhibit the Na,K-ATPase activity in old rats. These findings are different from what has been reported in terms of defective dopamine response in the PT of SHR compared with normotensive WKY rats (13). We found that although dopamine inhibits Na,K-ATPase in WKY rats but not in SHR, PDBu causes inhibition of the enzyme activity of similar extent in both SHR and WKY

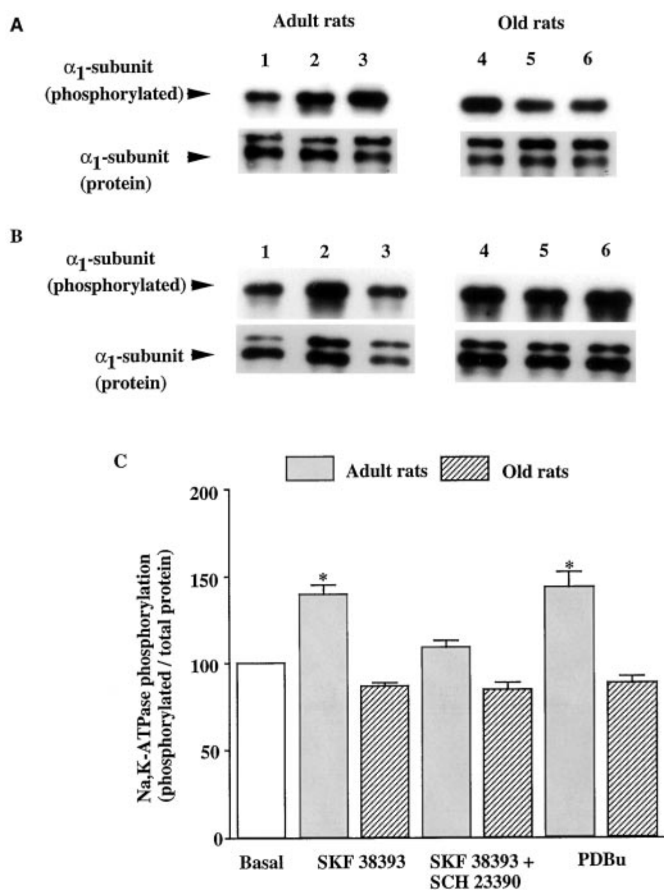


Figure 3. Effect of SKF 38393 or PDBu on phosphorylation of Na,K-ATPase in the cortex slices of adult and old rats. α_1-subunit of Na,K-ATPase antibody immunoprecipitated samples (10 μl) were immunodetected for Na,K-ATPase phosphorylation (see Materials and Methods section). Phosphorylation of Na,K-ATPase was measured as the ratio of arbitrary density unit of phosphorylated and the total protein of Na,K-ATPase in the immunoprecipitate. (A) Vehicle (lanes 1, and 4), SKF 38393 (10 μM; lanes 2, and 5), and PDBu (1 μM; lanes 3, and 6), representative blot of $n = 4$. (B) Vehicle (lanes 1, and 4), SKF 38393 (10 μM; lanes 2, and 5), and SKF 38393+SCH 23390 (10 μM each; lanes 3, and 6), representative blot of $n = 3$. (C) Data from A and B are plotted as percentage of basal, mean ± SEM. *, significant difference from basal (*t* test, $P < 0.05$).

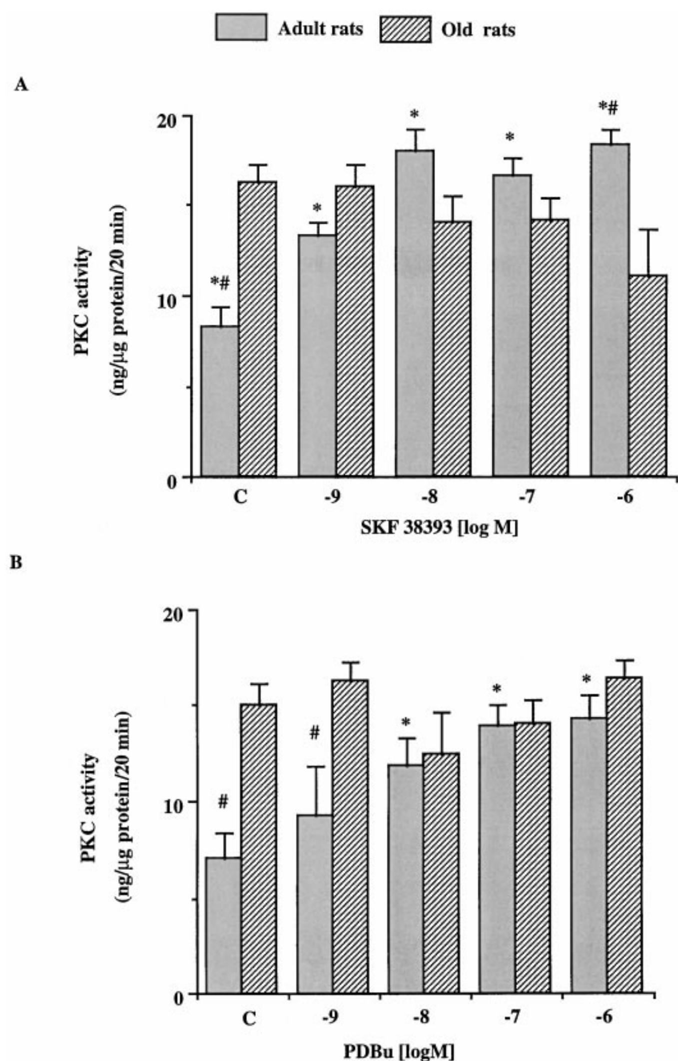


Figure 4. Effect of SKF 38393 (A) and PDBu (B) on protein kinase C (PKC) activity in the PT of adult and old rats. Basal activity of PKC in old rats was approximately twofold greater than in adult rats (adult *versus* old: 7.3 ± 1.1 *versus* 15.2 ± 2.6 phosphorylated peptide ng/μg protein per 20 min). The values are mean ± SEM; $n = 5$ to 6. *, significant difference within the group (one-way ANOVA, $P < 0.05$); #, significant difference between groups (*t* test, $P < 0.05$).

rats (13), suggesting that the defect is located primarily at the level of D_1-like receptor and not of Na,K-ATPase. Therefore, in the present study, we conducted additional experiments to identify the nature of the defect at the level of Na,K-ATPase in old rats. Inasmuch as it is the phosphorylation of the α_1-subunit of Na,K-ATPase that leads to the inhibition of the enzyme activity (27), we measured the basal phosphorylation state of the α_1-subunit of Na,K-ATPase as well as resting enzyme activity and found an elevation in the basal phosphorylation state and a decrease in the resting enzyme activity in old rats. We found that both SKF 38393 and PDBu did not cause any phosphorylation or inhibition of Na,K-ATPase in old rats. The most likely explanation for this finding is that the enzyme was already phosphorylated and inhibited under resting state, and therefore addition of either SKF 38393 or PDBu could not produce any further increase in phosphorylation in old rats. It should be noted that the total immunodetectable Na,K-ATPase protein content in the cortical membranes of old rats was not different from adult rats. This suggests that the decrease in Na,K-ATPase activity was due not to a decrease in Na,K-ATPase protein but rather to an increase in the basal phosphorylation of the enzyme. It is believed that phosphorylation of Na,K-ATPase causes a decrease in the affinity of sodium with the enzyme leading to a decrease in its activity. The basal enzyme activity was measured in the cortical membranes in the presence of 5 and 75 mM sodium. At 5 mM sodium, the decrease in basal activity in old compared with adult rats was greater (42%) than the decrease (25%) in the activity measured at 75 mM sodium (Vmax concentration). This observation further supports the suggestion that it is the phosphorylation of

the enzyme that caused a reduction in the basal activity of Na,K-ATPase in old rats. Phosphorylation has also been implicated as a prerequisite of internalization and finally inhibition of the enzyme activity (27,28). Because we found a decrease in the basal activity of Na,K-ATPase without alteration in its immunodetectable protein content in the membranes of old rats, it is suggested that the increased phosphorylation may have prevented Na,K-ATPase from further inhibition by SKF 38393 and PDBu. Whether the reduction in the basal activity of Na,K-ATPase has physiologic significance in the normal process of aging and maintaining electrolyte homeostasis remains to be determined. However, while the present research was in progress, published was a study that reported a decrease in Na,K-ATPase activity without a change in the immunodetectable protein content in the PT of old (30 mo) compared with adult (4 mo) Fischer 344 rats (29).

To explore the mechanism responsible for an increased basal phosphorylation of Na,K-ATPase in old rats, we measured PKC activity in the presence of SKF 38393 and PDBu. We found that although SKF 38393 and PDBu stimulated PKC activity in the PT of adult rats, these agents completely failed to stimulate the enzyme in old rats. This could be because the basal activity of PKC in old rats was similar to the stimulated activity in adult rats. Higher basal activity of PKC probably is responsible for the increased phosphorylation and the decreased activity of Na,K-ATPase in old rats. As mentioned earlier, while the activation of D_1-like receptor failed to produce inhibition of Na,K-ATPase activity in SHR, PDBu-mediated inhibition of the enzyme was not altered compared with WKY rats (13). In addition, the basal activity of PKC as well as of Na,K-ATPase in the PT of SHR was similar to WKY rats (13). As it relates to the old rats, it is important to identify further which isoforms of PKC that may have caused an overall increase in the basal activity of PKC in old rats are upregulated. In SHR, although there was no difference in the basal activity of PKC, there was a differential regulation of certain PKC isoforms (δ- and ζ-isoform), which were linked to the decreased ability of dopamine to promote natriuresis (17). Differential regulation of PKC isoforms and the higher activity of PKC have been reported in other tissues, *e.g.*, adipose tissue affecting insulin-mediated lipolysis in old rats (30), and in the heart and kidney of diabetic animals, where it was suggested to be linked to impaired function of endothelium, capillaries, and arterioles (31).

In summary, we found that in old rats, D_1-like receptor activation of PKC or direct activation of the enzyme with PDBu fails to cause the phosphorylation and subsequent inhibition of Na,K-ATPase. This phenomenon most likely is due to the increase in the basal PKC activity in old rats. We conclude that such an abnormality contributes to the failure of dopamine and D_1-like receptor agonist to inhibit Na,K-ATPase and subsequently increase sodium excretion in old rats.

Acknowledgments

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