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D_{1A} Dopamine Receptor Stimulation Inhibits Na^+/K^+-ATPase Activity through Protein Kinase A

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SUMMARY

We stably expressed the rat D_{1A} dopamine receptor in mouse fibroblast LTK^- cells and obtained specific ligand binding and functional activity characteristic of the D_{1A} dopamine receptor coupled to stimulation of adenylyl cyclase. In the transfected cells, the selective D_1 agonist fenoldopam caused a concentration-dependent inhibition of Na^+/K^+-ATPase activity, achieving maximum inhibition of ~30%. The latter was abolished by the selective D_1 antagonist (+)-SCH 23390 and by the specific protein kinase A inhibitor protein kinase inhibitor-(6–22) amide. In the nontransfected cells, fenoldopam did not affect Na^+/K^+-ATPase activity. 8-Chlorophenylthio-cAMP inhibited Na^+/K^+-ATPase activity in both transfected and nontransfected cells; this effect was blocked by protein kinase inhibitor-(6–22). These results indicate that the inhibition of Na^+/K^+-ATPase activity induced by agonist occupancy of D_{1A} receptors is mediated by protein kinase A.

Dopamine has been shown to inhibit the activity of Na^+/K^+-ATPase in neostriatal neurons (1) and in segments of the renal nephron such as the proximal tubule (2, 3) and the cortical collecting duct (4, 5). The selective D_1 dopamine receptor agonist fenoldopam produces a significant inhibition of Na^+/K^+-ATPase activity in the cortical collecting duct (5) and the thick ascending limb of the loop of Henle (6) via stimulation of protein kinase A. The observations that cAMP (or a cAMP analogue) itself decreases Na^+/K^+-ATPase activity in the renal cortical collecting duct (5), liver (7), brain (8), and pancreatic islets (9) support the concept that the adenylyl cyclase system and Na^+/K^+-ATPase are regulated reciprocally in various tissues. On the other hand, dopamine has been reported to inhibit Na^+/K^+-ATPase activity in isolated striatal neurons (1) and renal proximal tubules (3), whereas neither fenoldopam nor dibutyryl-cAMP alone had an effect on pump activity (1, 3). However, the relationship between the D_1 receptors and Na^+/K^+-ATPase has been assessed in tissues with a mixed population of dopamine receptors. Molecular biological advances have thus far revealed the presence of at least five different receptors (10). The D_{1A} and D_{1B} (D_5) receptors have been identified as the D_1 receptor subfamily linked to activation of adenylyl cyclase (10–12). We sought to determine whether agonist occupancy of the D_{1A} receptor, independently of other receptors, inhibits Na^+/K^+-ATPase activity. We also studied the role of the protein kinase A pathway in the observed effects.

We use as a model system stably transfected mouse fibroblast LTK^- cells expressing recombinant rat D_{1A} dopamine receptors. LTK^- cells were chosen because they do not have D_1 or D_2 receptors, as determined by radioligand binding and adenylyl cyclase measurements, but express a relatively ouabain-resistant Na^+/K^+-ATPase that is similar to that found in the rat kidney (11, 13, 14). Thus, any effect of dopaminergic stimulation should be due to activation of the transfected D_{1A} receptors. Nontransfected cells were used as controls.

Experimental Procedures

Materials. The drugs and reagents used were obtained from the following companies: [^{3}H]SCH 23390, DuPont-New England Nuclear (Boston, MA); fenoldopam, SmithKline Beecham (Philadelphia, PA); (+)-SKF 38393, (+)-SCH 23390, (*cis*)-flupenthixol, and apomorphine, Research Biochemicals Inc. (Natick, MA); PKI-(6–22) amide, a protein kinase A inhibitor, GIBCO Laboratories (Grand Island, NY); 8-CPT-cAMP, Sigma Chemical Co. (St. Louis, MO); and [γ-^{32}P]ATP, Amersham (Arlington Heights, IL). All other chemicals were of reagent grade and were obtained from standard suppliers.

Transfection and cell culture. A D_{1A} dopamine receptor cDNA (12) was subcloned in the expression vector pRc/CMV (Invitrogen, San Diego, CA) at the *Xba*I site. The resulting construct was used to stably

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ABBREVIATIONS: PKI-(6–22) amide, protein kinase inhibitor-(6–22) amide; 8-CPT-cAMP, 8-(chlorophenylthio)-adenosine 3′, 5′ cyclic monophosphate.

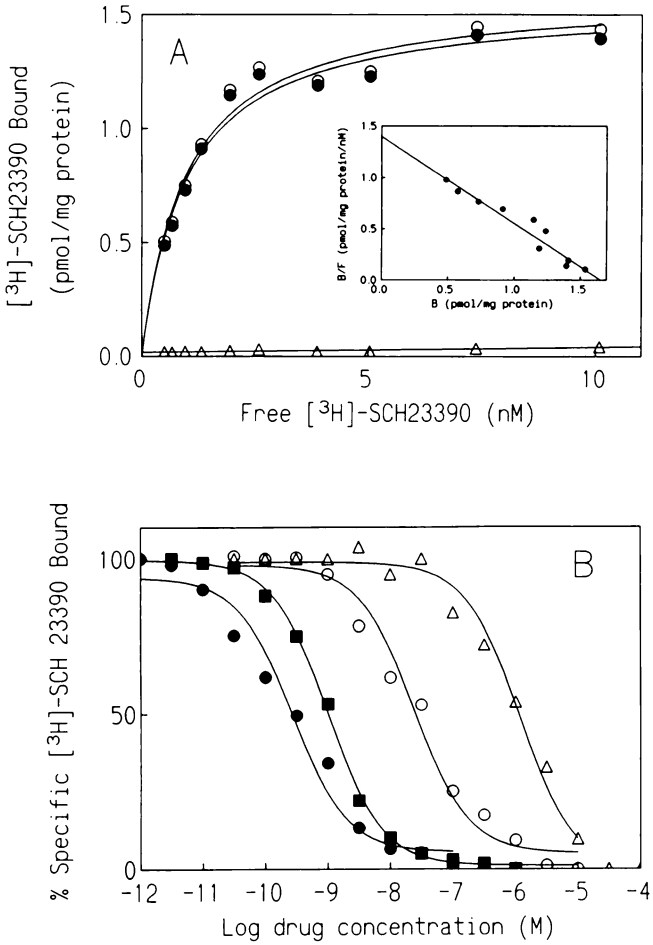

Fig. 1. Expression of the D_{1A} receptor cDNA in LTK$^-$ cells, assayed by [^{3}H]SCH 23390 binding. A, Saturation isotherms of the total (○), nonspecific (△), and specific (●) binding of [^{3}H]SCH 23390 to transfected LTK$^-$ cell membranes. *Inset*, Scatchard transformation of the specific binding data; *B*, bound; *F*, free. B, Competition of dopaminergic ligands for [^{3}H] SCH 23390 binding in D_{1A}-transfected LTK$^-$ cell membranes. In this experiment, [^{3}H]SCH 23390 (4 nM) was incubated with various concentrations of the following ligands: (+)-SCH 23390 (●), (*cis*)-flupenthixol (■), fenoldopam (○), and (−)-apomorphine (△). Average K_i and standard error values from three separate experiments are given in the text.

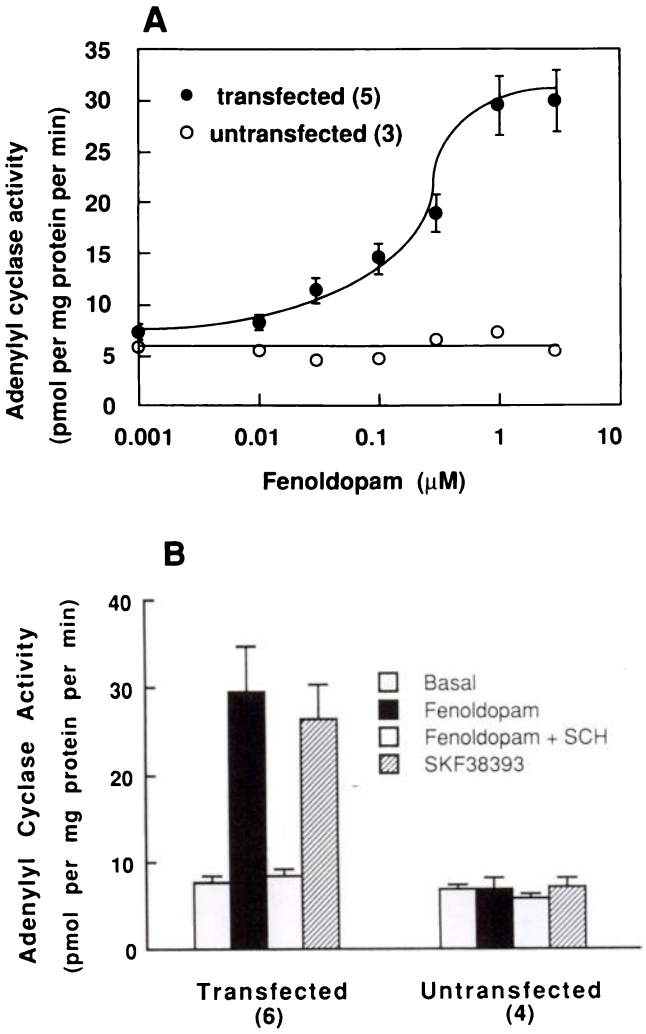

Fig. 2. A, Effect of various concentrations of fenoldopam on adenylyl cyclase activity in membranes from D_{1A}-transfected and nontransfected LTK$^-$ cells. B, Effect of fenoldopam (1 μM) and (+)-SKF 38393 (1 μM) on adenylyl cyclase activity in transfected and nontransfected cell membranes and the blocking effect of (+)-SCH 23390 (*SCH*) (1 μM). Values represent means ± standard errors. *Numbers in parentheses*, number of experiments.

transfect mouse fibroblast LTK$^-$ cells by a modified calcium phosphate method (15). The detailed methods for cell culture were described previously (14). The transfected cells were selected in a medium containing G418 sulfate (500 μg/ml; GIBCO) and were screened for D_{1A} receptor expression by using radioligand binding (see below).

Membrane preparation and receptor assays. Both transfected and nontransfected LTK$^-$ cells were treated by addition of 10 mM butyrate to the medium for about 48 hr before each experiment (16). Butyrate at 10 mM markedly enhanced the expression of the D_{1A} receptor and generally increased Na^+/K^+-ATPase activity by 10–20% in LTK$^-$ cells (data not shown). LTK$^-$ cell membranes were prepared by lysis of cell monolayers in 1 mM Tris, pH 7.5, for 15 min. The cell lysates were then scraped from the dish and centrifuged at 40,000 × *g* for 15 min. The pellet was resuspended in TME buffer (75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA). Ligand binding activity was measured using [^{3}H]SCH 23390 as described (17). Adenylyl cyclase activity was determined as described previously (17).

Determination of Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activity was measured by the method of Xie *et al.* (18). The membrane preparations from transfected or nontransfected cells were preincubated for 30 min at room temperature with vehicle (control) or with the selective D_1 agonist fenoldopam, the selective D_1 antagonist (+)-SCH 23390, fenoldopam plus (+)-SCH 23390, PKI-(6–22) amide (protein kinase A inhibitor), fenoldopam plus PKI-(6–22) amide, 8-CPT-cAMP, or 8-CPT-cAMP plus PKI-(6–22). Incubations were performed with [γ^{32}P]ATP for 30 min at 37°, with or without 1 mM ouabain. Each reaction was terminated by the addition of 1 ml of 8% perchloric acid. Released $^{32}P_i$ was converted to phosphomolybdate, extracted into 2-methylpropanol, and counted. Na^+/K^+-ATPase activity was calculated as the difference between the means of triplicate determinations of total and ouabain-insensitive hydrolyzed $^{32}P_i$/mg of protein/30 min, and the results are shown as percentage of control. Protein concentrations were determined according to the method of Lowry *et al.* (19).

Data analysis. Data are expressed as mean ± standard error. The dissociation constant (K_d), maximum receptor density (B_{max}), and inhibition constant (K_i) were calculated using nonlinear regression (GraphPAD InPlot version 3.14; GraphPAD Software, Inc., San Diego, CA). Significant differences ($p < 0.05$) were determined using Student's *t* test when two groups were compared and analysis of variance followed by the Bonferroni correction when more than two groups were compared.

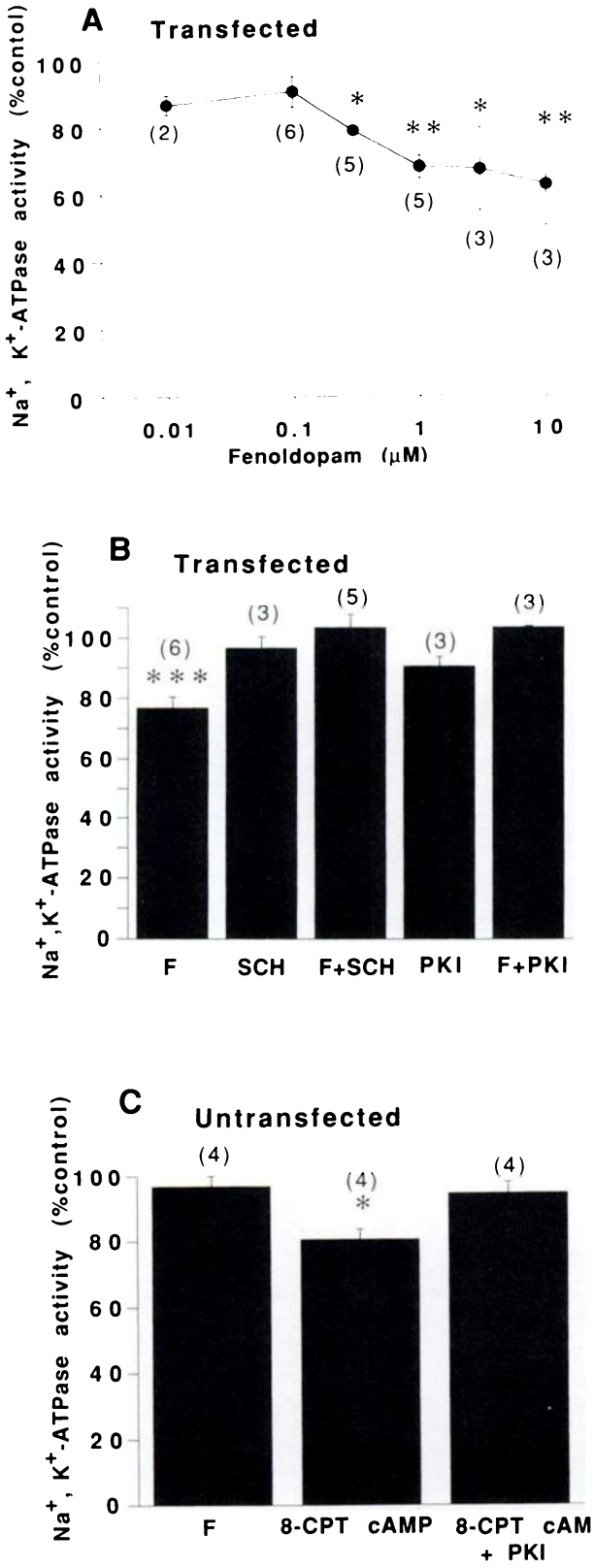

Fig. 3. Inhibition of Na^+/K^+-ATPase activity in D_{1A}-transfected and nontransfected cells by various drugs. Cell membranes were preincubated for 30 min with drugs, as follows: *F*, fenoldopam (1 μM); *SCH*, (+)-SCH 23390 (1 μM); *F+SCH*, fenoldopam (1 μM) plus (+)-SCH 23390 (1 μM); *PKI*, PKI-(6–22) (1 μM); *F+PKI*, fenoldopam (1 μM) plus PKI-(6–22) (1 μM); *8-CPT cAMP*, 8-CPT-cAMP (50 μM); *8-CPT cAMP+PKI*, 8-CPT-cAMP (50 μM) plus PKI-(6–22) (10 μM). All samples were compared with control samples incubated without drugs for 30 min (A, control activity = 1.1 ± 0.03 μmol of P_i/mg of protein/30 min, six experiments; B, control activity = 1.1 ± 0.05 μmol of P_i/mg of protein/30 min, seven experiments; C, control activity = 1.1 ± 0.08 μmol of P_i/mg of protein/30 min, eight experiments). Values represent means ± standard errors. *Numbers in parentheses*, number of experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control.

Results

Fig. 1A shows that [^{3}H]SCH 23390, a selective radiolabeled dopamine D_1 antagonist, specifically bound to transfected LTK^- cell membranes in a saturable fashion, with a B_{max} of 1.5 ± 0.2 pmol/mg of protein (four experiments) and a K_d of 1.1 ± 0.04 nM (four experiments). The K_d value is comparable to that for rat striatal D_{1A} receptors (20). No specific binding was detected in LTK^- cells that had not been transfected with pRcCMV-D_{1A}. Fig. 1B demonstrates the ability of several dopaminergic ligands to compete with specific [^{3}H]SCH 23390 binding to D_{1A}-transfected LTK^- cell membranes. (+)-SCH 23390 was the most potent agent (K_i = 0.5 ± 0.2 nM, three experiments). The nonselective dopaminergic antagonist (*cis*)-flupenthixol also exhibited high affinity (K_i = 3.2 ± 1.0 nM, three experiments). The K_i values for the selective D_1 agonist fenoldopam and the nonselective dopaminergic agonist (−)-apomorphine were 99.7 ± 6.9 nM (three experiments) and 620 ± 35 nM (three experiments), respectively. The rank order of potency and K_i values of these compounds are in agreement with those reported for D_{1A} receptors from rat striatum (20).

pRcCMV-D_{1A}-transfected LTK^- cells also exhibited D_1 receptor-mediated stimulation of cAMP production. When exposed to fenoldopam, nontransfected cells showed no increase in adenylyl cyclase activity (Fig. 2A). In contrast, transfected cells displayed a concentration-dependent and saturable increase in adenylyl cyclase activity, with a half-maximal stimulatory concentration (EC_{50}) of about 0.1 μM (Fig. 2A). This value is comparable to the previously reported values for canine striatum and bovine parathyroid D_1 receptors (20, 21) and transfected human D_{1A} receptors in human embryonic kidney 293 cells (22). The stimulatory effect of fenoldopam (1 μM) on adenylyl cyclase activity was blocked by 1 μM (+)-SCH 23390, a selective D_1 antagonist (Fig. 2B). (+)-SKF 38393, another selective D_1 agonist, also caused a marked increase in adenylyl cyclase activity. In nontransfected cells, (+)-SKF 38393 (1 μM) had no effect on adenylyl cyclase activity. These results indicate that the cloned D_{1A} receptor is positively linked to adenylyl cyclase.

In the D_{1A}-transfected cells, Na^+/K^+-ATPase activity in control samples averaged 1.1 ± 0.04 μmol of P_i/mg of protein/30 min (13 experiments). Ouabain-insensitive ATP hydrolysis averaged 1.1 ± 0.15 μmol of P_i/mg of protein/30 min (13 experiments) and was unaffected by any of the drugs. Fenoldopam produced a dose-dependent inhibition of Na^+/K^+-ATPase activity, with a maximal inhibition of about 30% at 1 μM (Fig. 3A). The inhibition was completely blocked by (+)-SCH 23390 (1 μM), which by itself had no effect (Fig. 3B). In addition, the fenoldopam-induced inhibition of Na^+/K^+-ATPase activity was abolished by the specific protein kinase A inhibitor PKI-(6–22) amide (1 μM) (Fig. 3B). In the nontransfected cells, fenoldopam (1 μM) had no effect on Na^+/K^+-ATPase activity (Fig. 3C) but the cAMP analogue 8-CPT-cAMP (50 μM) decreased Na^+/K^+-ATPase activity to 80.5 ± 3.2% of the control value (Fig. 3C).

The 8-CPT-cAMP-induced inhibition of Na^+/K^+-ATPase activity was abolished by PKI-(6–22) amide (10 μM).

Discussion

We stably expressed the rat D_{1A} dopamine receptor in LTK^- cells in order to assess the relationship between the D_{1A} receptor and Na^+/K^+-ATPase. This approach provides several advantages. First, it allows assessment of the D_{1A} receptor without the potential difficulties encountered when tissues with a mixed population of dopamine receptors are used. In such tissues, the relationship among these receptors and Na^+/K^+-ATPase can be examined only by the use of specific agonists and antagonists. Drugs that are truly specific for a particular receptor, however, do not exist. The inhibition of Na^+/K^+-ATPase activity by fenoldopam and (+)-SKF 38392 in the transfected cells suggests that the D_{1A} receptor-associated signaling pathway alone can regulate Na^+/K^+-ATPase, independently of other dopamine receptor-mediated signaling pathways.

Rat and human D_{1A} receptors expressed in COS-7 cells, LTK^- cells, or human embryonic kidney 293 cells are linked to activation of adenylyl cyclase activity but do not stimulate phosphatidylinositol turnover (11, 12, 22). We have also found that fenoldopam (1–10 μM) does not stimulate Ca^{2+} mobilization in our transfected LTK^- cells.[1] Therefore, the inhibition of Na^+/K^+-ATPase activity by agonist occupation of D_{1A} receptors in transfected LTK^- cells is independent of changes in intracellular calcium (and presumably of the phosphatidylinositol pathway).

The present study is consistent with the observation that a D_1 agonist alone inhibits Na^+/K^+-ATPase activity in the cortical collecting duct (5) and the thick ascending limb of the loop of Henle (6), an effect mediated by activation of protein kinase A. The inhibitory effect of fenoldopam on Na^+/K^+ ATPase activity in LTK^- cells is similar to that reported by Meister *et al.* in the medullary thick ascending limb of Henle (35%); the inhibitory effect of 10 μM fenoldopam ranged from 35% to 60% (4–6, 23). However, stimulation of D_1 receptors alone did not affect Na^+/K^+-ATPase activity in isolated striatal neurons (1) and renal proximal convoluted tubules (3, 4, 23). It has also been reported that dopamine-induced inhibition of Na^+/K^+-ATPase activity in proximal tubules is mediated by protein kinase C (24). These discrepancies may be the result of differences in the tissues (e.g., abundance of D_{1A} receptors and interaction with other receptors). In the current study, the relatively high level of D_{1A} receptor expression in LTK^- cells may have facilitated the uncovering of the inhibitory effect of the D_{1A} signaling pathway on Na^+/K^+-ATPase activity.

The present study shows that the selective D_1 agonist decreases Na^+/K^+-ATPase activity by approximately 30% in D_{1A} receptor-transfected LTK^- cells. This action is dependent on the activation of protein kinase A, because the D_1 agonist-induced inhibition of Na^+/K^+-ATPase activity was completely abolished by a specific inhibitor of protein kinase A. In addition, the cAMP analogue 8-CPT-cAMP inhibited Na^+/K^+-ATPase activity in both nontransfected (Fig. 3C) and transfected (data not shown) cells. This inhibitory action was also completely blocked by a protein kinase A inhibitor (Fig. 3C).

In summary, our results indicate that agonist occupancy of D_{1A} receptors causes up to 30% inhibition of Na^+/K^+-ATPase activity, via the protein kinase A pathway, independently of D_2 or other dopamine receptor-associated signaling mechanisms. We suggest that the occupation of the D_{1A} receptor results in the stimulation of adenylyl cyclase activity; the cAMP thus produced phosphorylates protein kinase A. Protein kinase A leads to the phosphorylation of Na^+/K^+ ATPase, which inhibits its activity (25). Protein kinase A also phosphorylates a dopamine-related phosphoprotein-32) and maintains Na^+/K^+ ATPase in its inhibited phosphorylated state by inhibiting protein phosphatase-1 (6).

[1] A. Horiuchi, K. Takeyasu, P. A. Jose, R. A. Felder, Unpublished observations.

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