Genistein Inhibits the Regulation of Active Sodium–Potassium Transport by Dopaminergic Agonists in Nonpigmented Ciliary Epithelium

Yosibyuki Nakai, 1,2 William L. Dean, 3 Yining Hou, 1 and Nicholas A. Delamere 1,4

PURPOSE. To determine whether dopamine receptor stimulation regulates Na,K-ATPase-mediated ion transport in cultured nonpigmented ciliary epithelium (NPE).

METHODS. Using a rabbit NPE cell line, active Na-K transport activity was determined by measuring ouabain-sensitive potassium (86Rb) uptake in cell monolayers. Western blot analysis of membrane material obtained from cell homogenates was conducted to examine tyrosine phosphorylation of membrane proteins.

RESULTS. Ouabain-sensitive potassium (86Rb) uptake was inhibited in the presence of either dopamine or the D1-selective agonist SKF82958. The response was suppressed by SCH23390, a D2 antagonist, but not by sulpiride, a D2-selective antagonist. Quinpirole, a D2-selective agonist, did not cause inhibition of ouabain-sensitive potassium (86Rb) uptake. Cyclic adenosine monophosphate (cAMP) was detectably increased in SKF82958-treated cells, although the concentration of SKF required to elevate cell cAMP was higher than the concentration needed to inhibit ouabain-sensitive potassium (86Rb) uptake. The protein kinase A inhibitor H89 prevented the 86Rb uptake response to SKF82958. Genistein, an inhibitor of tyrosine kinases, also prevented the 86Rb uptake response to SKF82958. Membrane material isolated from cells exposed to SKF82958 showed an increase in the density of several phosphotyrosine bands. These changes in phosphotyrosine immunoblot density were not observed in material isolated from cells that received either genistein or SCH23390 before SKF82958 treatment.

CONCLUSIONS. The results of this study suggest D1 agonists cause a reduction of Na,K-ATPase-mediated ion transport by a mechanism that could involve a tyrosine kinase step. (Invest Ophthalmol Vis Sci. 1999;40:1460–1466)
In the present study we report evidence suggesting activation of D1 receptors caused a reduction of active sodium-potassium transport in cultured rabbit NPE. Judged by the ability of genistein and herbinicin to blunt the change of active sodium-potassium transport, we suggest that activation of a tyrosine kinase could be one of the steps in the mechanism that leads to sodium pump inhibition after D1 receptor activation.

METHODS

Chemicals

86Rb-Cl was purchased from NEN (Boston, MA). SKF82958, SCH23390, quinpirole, and sulpiride were obtained from Research Biochemicals (Natick, MA). N-[2((p-Bromopconannyl)-amino)ethyl]-5-isoquinolinesulfonamide, HCl, (H89), was obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell Culture

The rabbit nonpigmented ciliary epithelial cell line used in this study was derived by transformation of a semiconfluent primary culture with wild-type simian virus, following standard procedure in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The cell line was a kind gift from Miguel Coca-Prados (Yale University, New Haven, CT) and has been used previously in studies of Na,K-ATPase,13 Na/K/2Cl cotransporter,14 and prostanoid receptors.15 The cell line and rabbit ciliary epithelium express similar Na,K-ATPase isoforms.13 The cells were grown at 37°C in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 8% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) under a humidified atmosphere of 5% CO2 and 95% air in a 25-cm2 tissue culture flask ( Falcon Primaria; Becton Dickinson, Oxnard, CA). When the cells became confluent approximately 3 to 4 days after seeding, they were trypsinized with 0.05%/0.02% trypsin/EDTA. The cells were passaged at a split ratio of 2:5. 86Rb uptake experiments were performed using confluent monolayers of cells grown on 24-well culture plates (Falcon Primaria; Becton Dickinson). Cells grown on 6-well culture plates were used for immunoblot studies.

Measurement of 86Rb Uptake

Active ion transport mediated by Na,K-ATPase was evaluated by measuring ouabain-sensitive 86Rb uptake. Rubidium is transported almost identically to potassium. Before the uptake experiments, the growing medium was replaced with serum-free DMEM for at least 2 hours. Uptake experiments were performed in Krebs solution containing 119 mM NaCl, 4.7 mM KCl, 1.1 mM KH2PO4, 1 mM MgCl2, 5.5 mM glucose, and 25 mM NaHCO3 (pH 7.4). The Krebs solution was equilibrated with 95% O2/5% CO2 and incubated for a specified period in Krebs solution that contained SKF82958. The Krebs solution also contained IBMX (2.5 mM), an inhibitor of phosphodiesterase, added to prevent cAMP breakdown. After the 15-minute period, the Krebs solution was tipped off and replaced with ice-cold Tris-EDTA buffer containing 50 mM Tris and 4 mM EDTA. After this, the cells were scraped from the plate and the mixture transferred to glass tubes in which material equivalent to at least 10 monolayers was pooled and boiled for 5 minutes. The mixture was centrifuged at 3000 rpm for 5 minutes and the supernatant assayed for cAMP.

Western Blot Analysis

Before western blot experiments, the growing medium was replaced by serum-free DMEM for at least 2 hours. The cell monolayers were then washed two times with Krebs solution and incubated for a specified period in Krebs solution containing test agents. After this, the cells were homogenized using a tissue grinder ( Kontes, Vineland, NJ) in a buffer containing 150 mM sucrose, 5 mM HEPES, 1 mM EDTA, and a mix of protease inhibitors ( Complete TM; Boehringer Mannheim, Mannheim, Germany). The homogenate was first centrifuged at 3000 rpm and the pellet discarded. The supernatant was then centrifuged at 140,000g for 60 minutes. Samples of the pellet material (30 µg protein) were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel then transferred electrophoretically to a nitrocellulose sheet that was blocked with 1% bovine serum albumin for 1 hour at room temperature. The nitrocellulose sheet was then incubated for a further 12 hours at 4°C with a mouse monoclonal antibody directed against phosphotyrosine ( Transduction Laboratories, Lexington, KY). After several washes, visualization of the phosphotyrosine immunoblot was made possible by incubating the nitrocellulose membrane with chemiluminescence substrate ( Amersham).

Data Analysis

Unless otherwise noted, statistical analysis was conducted using Student’s t-test. P < 0.05 was considered to indicate a significant difference.

RESULTS

Dopamine inhibited ouabain-sensitive potassium 86Rb uptake by cultured rabbit nonpigmented ciliary epithelium: Added 15 minutes before the measurement of 86Rb uptake, 1 µM dopamine reduced the rate of ouabain-sensitive potassium 86Rb uptake to approximately 25% of the control value (Fig. 1). To
the mechanism by which dopamine causes inhibition of ouabain-sensitive potassium (86Rb) uptake. In keeping with this notion, significant inhibition of ouabain-sensitive potassium (86Rb) uptake was observed in cells exposed to the D2-selective agonist SKF82958 (0.1 μM; Fig. 1C). Furthermore, the inhibitory effect of SKF82958 was abolished by the D2-selective antagonist SCH23390. In cells exposed to the D2-selective agonist quinpirole (0.1-10 μM) no detectable change of ouabain-sensitive potassium (86Rb) uptake rate was observed. The uptake rate measured in the presence of 10 μM quinpirole, for example, was 104.3% ± 26.7% of the control value (mean ± SE; n = 8).

Because Na,K-ATPase-mediated outward transport of sodium ions balances the inward movement of sodium, we considered the possibility that the SKF82958-induced reduction in the rate of active sodium-potassium transport could be secondary to a reduction in the rate of inward sodium movement. A set of experiments was conducted in which cells were exposed to SKF82958 in the presence of amphotericin B, a pseudoionophore that effectively increases cation permeability to cause an increase in the inward leak rate for sodium. Importantly, the inhibitory effect of SKF82958 persisted in amphotericin B-treated cells; the ouabain-sensitive potassium (86Rb) uptake rate measured in cells exposed to both SKF82958 (0.1 μM) and amphotericin B (1 μM) was 93.6 ± 14.1 nanomoles potassium accumulated per milligram protein per 15 minutes, which was significantly (P < 0.01) less than the rate of 138.9 ± 9.8 nanomoles measured in cells that received amphotericin B alone (mean ± SE; n = 16). As expected, the baseline rate of ouabain-sensitive potassium (86Rb) uptake measured in the presence of amphotericin B was significantly higher than the baseline rate of 80.9 ± 10.7 nanomoles potassium accumulated per milligram protein per 15 minutes measured in the absence of amphotericin B. The increase in the baseline rate of ouabain-sensitive potassium (86Rb) uptake almost certainly stemmed from the increased inward sodium leak, which tended to increase the cytoplasmic sodium concentration in cells that received amphotericin B.

In many tissues, D1 receptors elicit cell responses by activation of a cAMP-protein kinase A pathway. To examine the possible involvement of cAMP-dependent mechanisms in the D1 agonist-induced inhibition of ouabain-sensitive potassium (86Rb) uptake, some cells received SKF82958 (0.1-10 μM) for 15 minutes, and cAMP was then measured. The amount of cAMP in the cell monolayers was significantly elevated in cells that received SKF82958 at a concentration of 3 μM or higher (Fig. 2). Although the concentration of SKF82958 required to elicit a measurable increase of cAMP was higher than the SKF82958 concentration needed to inhibit ouabain-sensitive potassium (86Rb) uptake, it should be noted that detection of cAMP was made difficult because of the small amount of cell material. The inhibitory effect of D1-selective antagonist SKF82958 on ouabain-sensitive potassium (86Rb) uptake was sensitive to the protein kinase A inhibitor H89. When H89 (10 μM) was applied to cell monolayers 15 minutes before the addition of SKF82958, the inhibitory effect of SKF82958 on ouabain-sensitive potassium (86Rb) uptake was abolished in the presence of H89 (Fig. 3). Added alone, H89 did not change the rate of ouabain-sensitive potassium (86Rb) uptake. In the presence of 10 μM H89, the rate was 109.7% ± 16.0% of the control rate.
Dopamine Inhibits NaK Transport 1463

**FIGURE 2.** The influence of SKF82958 on cell cAMP content. Cell monolayers were exposed to SKF82958 (0.1–10 μM) for 15 minutes before measurement of cAMP. Control cells did not receive SKF82958. The data are presented as picomoles per milligram protein and are the mean of results from six experiments. SE is indicated by a vertical bar. *Significant difference from control; P < 0.01.

The results of studies with H89 suggest the possible involvement of protein kinase A in the mechanism that leads to a change of active sodium-potassium transport rate in SKF82958-treated cells. Interestingly, the tyrosine kinase inhibitor genistein also inhibited the D₃ agonist-induced reduction of ouabain-sensitive potassium (⁸⁶Rb) uptake. Added 20 minutes before the D₃ agonist, genistein (100 μM) abolished the inhibitory effect of SKF82958 on ouabain-sensitive potassium (⁸⁶Rb) uptake (Fig. 4). Added alone, genistein did not significantly alter the rate of ouabain-sensitive potassium (⁸⁶Rb) uptake. In the presence of 100 μM genistein, the rate was 116.5% ± 10.4% of the control rate (mean ± SE; n = 6). Because genistein is known to inhibit several protein kinases, experiments were carried out to confirm that a different tyrosine kinase inhibitor, herbimycin, inhibits the D₃ agonist-induced reduction of ouabain-sensitive potassium (⁸⁶Rb) uptake. In the presence of 5 μM herbimycin added 15 minutes before adding 0.1 μM SKF82958, the rate of ouabain-sensitive potassium (⁸⁶Rb) uptake measured 15 minutes later was 144.1 ± 19.1 nanomoles potassium accumulated per milligram protein per 15 minutes, which was not significantly different from the paired control uptake rate of 182.0 ± 21.0 nanomoles (mean ± SE; n = 12).

The above studies with genistein and herbimycin suggest tyrosine kinases could be activated in cells exposed to SKF82958. To confirm this, western blot experiments were conducted to search for evidence of a possible increase in tyrosine phosphorylation of membrane proteins in cells exposed to SKF82958. Immunoblots of membrane material isolated from cells that received 0.1 μM SKF82958 revealed an increased density of several phosphotyrosine immunoreactive polypeptide bands (Fig. 5). Importantly, the SKF82958-induced increase in phosphotyrosine immunoreactive band densities was not observed in cells that were exposed to SKF82958 in the presence of genistein (100 μM). Evidence of D₃ receptor involvement in the chain of events leading to the increase of tyrosine phosphoprotein immunoreactive band densities in SKF82958-treated cells was provided by the finding that the increases in band density were not detected in immunoblots of membrane material isolated from cells that received SKF82958 in the presence of the D₃ antagonist SCH23390.

**DISCUSSION**

Dopamine was found to reduce the rate of ouabain-sensitive potassium (⁸⁶Rb) uptake in cultured rabbit NPE. Based on the
The apparent involvement of the D₂-like receptor is consistent with an earlier report on the identification of D₂ receptors in tissues such as kidney cortical collecting duct and thick ascending limb of the loop of Henle and in Madin-Darby canine kidney (MDCK) cells. It has been found that selective activation of D₂ receptors elicits Na⁺K⁺ATPase inhibition in tissues such as kidney cortical collecting duct and thick ascending limb of the loop of Henle and in Madin-Darby canine kidney (MDCK) cells. The involvement of the D₁-like receptor subtype in the Na⁺K⁺ATPase responses to dopamine fits well with the present study, in which SKF82958, a D₁-selective agonist, was found to cause marked inhibition of active sodium-potassium transport mediated by Na⁺K⁺Cl⁻ cotransport in cultured fetal human NPE cells. In that study, 10 μM dopamine failed to change the rate of Na⁺K⁺-ATPase activity in NPE cells after D₁ or D₂ agonist is added alone. A similar response pattern is found in kidney proximal tubule, in which it has been suggested that Na⁺,K⁺-ATPase is inhibited by a synergistic action of both D₁ and D₂ receptors.

In a recent study, Riese et al. presented evidence that indicates that activation of D₁-like receptors causes stimulation of Na⁺⁺K⁺Cl⁻ cotransport in cultured fetal human NPE cells. In that study, 10 μM dopamine failed to change the rate of Na⁺,K⁺-ATPase-mediated ⁸⁶Rb uptake. This absence of a sodium pump response to dopamine may be the result of species differences between the human-derived cells used by Riese et al. and the rabbit-derived cells used in the present study. Differences between the sodium pump responses to dopamine detected by Riese et al. and those reported here could also be a consequence of differences in experimental design. Riese et al. conducted ⁸⁶Rb uptake experiments in cells exposed continuously to 1 μM indomethacin. It has been reported earlier that indomethacin alters some Na⁺,K⁺-ATPase responses in cultured NPE cells.

Dopamine receptors of the D₁ subtype generally elicit cell responses by mechanisms involving elevation of cAMP and activation of protein kinase A, and indeed, the receptor was initially characterized on the basis of its ability to activate adenyl cyclase in response to dopamine. The evidence for the involvement of this pathway in the D₁ agonist-induced reduction of active sodium-potassium transport in the NPE cell is not entirely conclusive. Although we detected an increase of the cAMP level in SKF82958-treated cells, the concentration of
SKF82958 required to elicit detectable cAMP elevation was higher than the concentration needed to cause inhibition of ouabain-sensitive potassium (\(^{86}\)Rb) uptake. Although this result appears to indicate that cAMP elevation may not be necessary for the SKF82958-induced change of ouabain-sensitive potassium (\(^{86}\)Rb) uptake to occur, it could also stem from technical limitations with cAMP measurements. Detection of cAMP was made difficult, particularly at low concentrations of SKF82958, because of the small amount of cell material and the relatively brief duration of exposure to the agonist. It should also be kept in mind that the concentration of cAMP detected in the whole cell may be an underestimate of the local concentration of cAMP adjacent to the plasma membrane location of Na,K-ATPase and other ion transport mechanisms. Support for the involvement of the cAMP-protein kinase A pathway in SKF82958-treated cells comes from the finding that the protein kinase A inhibitor H89 abolished the reduction of ouabain-sensitive potassium (\(^{86}\)Rb) uptake caused by SKF82958. Although other investigators have found evidence suggesting a role for protein kinase A in Na,K-ATPase responses to dopamine, it has also been suggested that activation of protein kinase C is a necessary event. This did not seem to be the case in the present study, because the protein kinase C-selective inhibitor calphostin C was not effective as an inhibitor of \(^{86}\)Rb uptake responses to the D\(_1\)-selective dopamine agonist SKF82958 (data not shown).

In human platelets it has been known for some time that plasma membrane calcium ATPase activity is subject to modulation by several cellular pathways including the cAMP-protein kinase A pathway. However, a recent report suggests activation of tyrosine kinases may lead to changes in plasma membrane calcium ATPase activity in the human platelet. The ability of both genistein and herbimycin to blunt the SKF82958-induced inhibition of sodium–potassium transport suggests a tyrosine kinase step may be involved in the chain of events that link activation of the D\(_1\) receptor to the change of the sodium pump activity in cultured rabbit NPE cells. Consistent with this notion, western blot experiments using an antibody directed against phosphotyrosine showed a marked increase in the density of a number of immunoreactive bands in membrane material isolated from cells that had been exposed to SKF82958. Importantly, there was no detectable increase in the density of phosphotyrosine immunoreactive bands in membrane material isolated from cells exposed to SKF82958 in the presence of either the tyrosine kinase inhibitor genistein or the D\(_1\) antagonist SCH23390. Taken together, these findings suggest that activation of the D\(_1\) receptor could lead to stimulation of tyrosine kinases. Our studies with genistein and herbimycin raise the possibility that activation of a tyrosine kinase could be a necessary part of the mechanism that leads to inhibition of active sodium–potassium transport after D\(_1\) receptor activation.

It is noteworthy that reduction of active sodium–potassium transport in cultured rabbit NPE cells is elicited by activation of the D\(_1\)-like receptor subtype. Activation of D\(_1\) receptors generally causes an increase of cAMP, but although Mancino et al. have suggested that D\(_1\) receptor activation could change the rate of aqueous humor formation, there is considerable uncertainty regarding the nature of the effects of elevated tissue cAMP on fluid production by the ciliary body. Whereas the adenylyl cyclase activator forskolin slows aqueous humor secretion, the rate of secretion is stimulated by beta adrenergic agonists that also activate adenyl cyclase. Slowing the rate of aqueous humor secretion leads to reduction of intraocular pressure, although such pressure reduction can also follow an increase in the rate of aqueous humor outflow from the eye. Reduction of intraocular pressure is desirable for the therapy of glaucoma. In conscious rabbits, dopamine has been reported to cause a mixed response; high concentrations of dopamine cause an increase in intraocular pressure, whereas low concentrations of dopamine reduce intraocular pressure.

In summary, we present evidence for a reduced rate of active sodium–potassium transport in cultured NPE cells exposed to dopamine, and we suggest that the mechanism of cation transport inhibition may involve a tyrosine kinase-mediated step. Because these experiments were performed using a virus-transformed cell line, the responses could be different from those in the native tissue. However, to our knowledge, there have not been widespread reports of possible linkage between tyrosine kinases and the actions of dopamine on the sodium pump mechanism. The results appear to justify future studies to determine whether D\(_1\) agonists elicit similar genistein-sensitive responses in native NPE cells or other epithelial tissues.

References


