Involvement of G protein-coupled receptor kinase 4 and 6 in rapid desensitization of dopamine D1 receptor in rat IEC-6 intestinal epithelial cells

Sónia Fraga, Pedro A. Jose, and Patrício Soares-da-Silva

Involvement of G protein-coupled receptor kinase 4 and 6 in rapid desensitization of dopamine D1 receptor in rat IEC-6 intestinal epithelial cells. *Am J Physiol Regul Integr Comp Physiol* 287: R772–R779, 2004. First published May 27, 2004; 10.1152/ajpregu.00208.2004.—Dopamine-induced inhibition of Na+/K+-ATPase has been suggested to play a role in the regulation of Na+ absorption at the intestinal level, and these effects were mediated by dopamine D1-like receptors. The aim of this work was to evaluate the effect of the activation of the D1-like receptors on the activity of the Na+/H+ exchanger (NHE) in the rat intestinal epithelial cell line IEC-6. The presence of D1 receptors was confirmed by immunoblotting. The dopamine D1-like receptor agonist SKF-38393 produced a concentration-dependent inhibition of NHE activity and stimulation of adenylyl cyclase (AC), this being antagonized by the D1 selective antagonist SKF-83566. Effects of SKF-38393 on NHE and AC activities were maximal at 5 min of exposure to the agonist and rapidly diminished with no effect at 25 min. Exposure of cells for 25 min to dibutyryl-cAMP (0.5 mM) or to the AC activator forskolin (3 μM) effectively inhibited NHE activity. Pretreatment of cells with heparin (1 μM), a nonspecific G protein-coupled receptor kinase (GRK) inhibitor, prevented the loss of effects on NHE activity after 25 min exposure to SKF-38393. The presence of GRK4, GRK6A, and GRK6B was confirmed by immunoblotting. Overnight treatment with the anti-GRK4–6 antibody complexed with Lipofectin was also effective in preventing loss of the effects of SKF-38393 on NHE and AC activities. It is concluded that dopamine D1 receptors in IEC-6 rapidly desensitize to D1-like agonist stimulation and GRK4 and 6 appear to be involved in agonist-mediated responsiveness and desensitization.

Na+/H+ exchanger; adenylyl cyclase

Dopamine receptors belong to the large family of G protein-coupled receptors and thus far, five distinct genes encoding different dopamine receptor proteins were isolated and characterized in mammals (4, 20, 30). These proteins can be subdivided into D1- and D2-like receptors that differ in their structural, pharmacological and transductional properties (12, 28). The D1-like receptors are generally coupled to Gs and their activation leads to a stimulation of adenylyl cyclase (AC) activity and increased levels of the second messenger cAMP. In jejunal cells, the dopamine inhibitory effect on Na+/K+-ATPase activity has been shown to be mediated by activation of this subclass of dopamine receptors (33); however, this effect was observed in young but not in adult rats (17, 32, 33). This absence of dopamine effect in adult jejunal cells may be explained by regulatory mechanisms that modulate signaling by G protein-coupled receptors such as agonist-induced desensitization of receptors. On the other hand, jejunal epithelial cells from spontaneous hypertensive rats, in contrast to their normotensive controls (Wistar-Kyoto rats), fail to respond to dopamine (18), as has been reported for the kidney. In genetic hypertension, the D1 receptor is uncoupled from its G protein complex, resulting in a decreased ability to regulate renal sodium transport. The impaired D1 receptor/G protein coupling in renal proximal tubules in genetic hypertension is secondary to abnormal phosphorylation and desensitization of the D1 receptor (14).

Phosphorylation is often involved in receptor desensitization and is mediated by two classes of serine/threonine kinases: the second messenger-dependent kinases, such as protein kinase A (PKA) and protein kinase C (PKC), or the specific kinases that phosphorylate the agonist-occupied or activated receptors and referred to as G protein-coupled receptor kinases (GRKs) (8, 15, 26). This family of kinases is composed of seven members (GRK1 to GRK7) and is widely expressed, which is suggestive of their important role in the regulation of G protein-coupled receptor responsiveness. Involvement of these kinases in agonist-induced desensitization of dopamine D1-like receptors has been previously reported. Indeed, intracellular inhibitors of kinases or elimination of potential phosphorylation sites in the receptors via site-directed mutagenesis could attenuate D1 receptor desensitization (13). Moreover, studies involving heterologously expressed D1 receptors in SF9 (21), HEK 293 (31), or CHO cells (6) have shown that the D1 receptor undergoes agonist-induced phosphorylation. In HEK 293 and CHO cells this phenomenon is enhanced by coexpression with GRKs 2, 3, 4, and 5 (6, 31). Recently, in renal proximal tube cells a role for GRK2 and GRK4 in the homologous desensitization of D1 receptors was reported (6, 34).

The aim of the present study was to evaluate the effect of the activation of the D1-like receptors on the activity of the Na+/H+ exchanger (NHE) in IEC-6 cells, a rat epithelial cell line that in culture has features of small intestinal crypt cells (27). It is reported that IEC-6 cells are endowed with dopamine D1 receptors, the stimulation of which results in inhibition of NHE activity and AC stimulation. These effects, however, rapidly diminished, but recovered after treatment of cells with the nonspecific GRK inhibitor heparin or the anti-GRK4–6 antibody. The dopamine D1-like-induced NHE inhibition is a cAMP-mediated event, but PKA is suggested not to be involved in receptor desensitization.

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Address for reprint requests and other correspondence: P. Soares-da-Silva, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal (E-mail: psoaresdasilva@netcabo.pt).

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Effect of GRK inhibitors. In this set of experiments designed to evaluate the role of GRKs in the desensitization mechanism, cells were preincubated overnight at 37°C in 5% CO₂-95% air atmosphere in the presence of heparin (1 μM) or monoclonal anti-GRK4–6 (1.4 μg/ml) (Sigma Chemical, St. Louis, MO) together with 5 μg/ml Lipofectin (Invitrogen, San Diego, CA) to facilitate their entry in cells (10, 34).

cAMP measurement. Total cAMP was determined with an enzyme immunoassay kit (Amersham Life, Arlington Heights, IL) according to manufacturer’s protocol. Briefly, IEC-6 cells were preincubated for 15 min at 37°C in Hanks’ medium (in mM: 137 NaCl, 5 KCl, 1.2 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 0.25 CaCl₂, 1.0 MgCl₂, 0.15 Tris·HCl, and 1.0 sodium butyrate, pH 7.4) containing 100 μM IBMX, a phosphodiesterase inhibitor. Cells were then incubated for 5 to 25 min with increasing concentrations of the D₁-like receptor selective agonist SKF-38393 (0.1–1 μM). The reaction was stopped by addition of 20 μl of lysis reagent (2.5% dodecyltrimethylammonium bromide in 0.05 M acetate buffer pH 5.8 containing 0.02% nonidet-p40). cAMP was then extracted and measured by specific enzyme immunoassay. Values are means of 8 experiments per group; vertical lines show SE.

Fig. 1. Assessment of intracellular pH (pHᵢ) during the Na⁺-dependent pHᵢ recovery after an acid load imposed by exposure to NH₄Cl followed by Na⁺ removal of the incubation medium in IEC-6 cells, in the absence and the presence of ethylisopropylamiloride (EIPA; 10 μM) or amiloride (1 mM). Symbols represent means of 8 experiments per group; vertical lines show SE.

Fig. 2. Effect of EIPA (0.01–10 μM; A) and amiloride (0.1–1 mM; B) on Na⁺/H⁺ exchanger (NHE) activity in IEC-6 cells. IC₅₀ values with intervals of 95% of confidence. Symbols represent the mean of 8 experiments per group; vertical lines show SE.
and aliquots were taken for measurement of total cAMP content.

Western blotting analysis. IEC-6 cells cultured to 90% of confluence were washed twice with PBS and total cell protein was extracted for dopamine D₁ receptor, NHE1, NHE3, GRK4, GRK6A, and GRK6B detection. Briefly, to obtain total cell extract, cells were lysed by brief sonication (15 s) in lysis buffer with protease inhibitors (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, and 2 μg/ml each aprotinin and leupeptin) and incubated on ice for 1 h. After centrifugation (16,000 g, 30 min, 4°C), the supernatant was collected and protein concentration was determined using the method of Bradford (3). Forty micrograms (NHE1 and NHE3), 25 μg (D₁ receptor), or 50 μg of protein (GRK4, GRK6A, and GRK6B) were mixed in 6× sample buffer (0.35 M Tris/HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromphenol blue) and boiled for 5 min. Proteins were subjected to SDS-PAGE (10% SDS-polyacrylamide gel) and electrotransfered onto nitrocellulose membranes. The transblot sheets were blocked with 5% of non-fat dry milk in 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20, overnight at 4°C. Then the membranes were incubated with appropriately diluted antibodies: rabbit anti-NHE1 or anti-NHE3 polyclonal antibodies (Alpha Diagnostics, Autogenbioclear, Wiltshire, UK), rabbit anti-dopamine receptor D₁ polyclonal antibody (Chemicon International, Temecula, CA), rabbit anti-human GRK4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-GRK6A and GRK6B (35). The reaction was detected by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and ECL system (Amersham Life).

Drugs. Amiloride, forskolin, H-89, low molecular weight heparin, SKF-38393 hydrochloride, SKF-83566 hydrochloride, and dibutyryl-cAMP were purchased from Sigma. BCECF-AM, ethylisopropropylamidole (EIPA), and nigericin were obtained from Molecular Probes (Eugene, OR). 4-Isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate (HOE-642), and 3-(54)-N-isopropylidene-2-methyl-acrylamide dihydrochloride (S-3226) were kindly provided by Dr. H. J. Lang from Aventis Pharma Deutschland (Frankfurt, Germany).

Data analysis. Geometric means are given with 95% confidence limits, and arithmetic means are given with SE. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons. A P value <0.05 was assumed to denote a significant difference. IC₅₀ values were determined from sigmoidal dose-response analysis using the GraphPad Prism statistics software.
RESULTS

In the present study, NHE activity was assayed in IEC-6 cells loaded with a pH-sensitive dye (BCECF), as the Na\(^+\)/H\(^+\) dependent recovery of pH measured after an acid load imposed by 20 mM NH\(_4\)Cl followed by removal of Na\(^+\)/H\(^+\) from the Krebs modified buffer solution, in the absence of CO\(_2\)/HCO\(_3\). As shown in Fig. 1, after acidification, IEC-6 cells showed a rapid alkalization on addition of 140 mM Na\(^+\)/H\(^+\). This alkalization process was largely inhibited by amiloride (1 mM) and by EIPA (10 \(\mu\)M), indicating that the endogenous NHE in IEC-6 cells is both an amiloride- and EIPA-inhibitable exchanger. The sensitivity of NHE to inhibition by amiloride and EIPA was also evaluated. Figure 2A shows inhibition of NHE activity in IEC-6 cells by both amiloride and EIPA, EIPA being considerably more potent than amiloride (IC\(_{50}\) values: amiloride = 54 \(\mu\)M; EIPA = 54 nM). Differences in sensitivity to inhibitors are in agreement with the observation that IEC-6 cells express both the EIPA-sensitive NHE3 subtype and the amiloride-sensitive NHE1 subtype (22, 23). The presence of both NHE1 and NHE3 isoforms was also confirmed by immunoblotting, as shown in Fig. 3. Immunoblotting studies also showed that this cell line endogenously expresses dopamine D\(_1\) receptors (Fig. 3). Because D\(_1\)-like receptors are generally coupled to G\(_s\) and their activation leads to a stimulation of AC activity and increased levels of the second messenger cAMP, it was decided to evaluate the effects of cAMP on NHE activity. For this purpose, IEC-6 cells were stimulated for 25 min with dibutyryl-cAMP (0.5 mM) or the AC activator forskolin (3 \(\mu\)M). Both compounds markedly reduced NHE activity (forskolin, 28 \(\pm\) 3\% decrease; dibutyryl-cAMP, 24 \(\pm\) 4\% decrease).

The activation of dopamine receptors by the selective D\(_1\)-like dopamine receptor agonist SKF-38393 markedly inhibited NHE activity (Fig. 5). D\(_1\)-like dopamine receptor antagonist SKF-83566 (1 \(\mu\)M) also significantly inhibited NHE activity (Fig. 6).
NHE activity (Fig. 4A). This inhibition was maximal at 5 min of exposure to the agonist (30%) and was progressively lost with the increase of exposure time to the agonist (10 min, 16% reduction; 25 min, 6% reduction). Similarly, the selective D₁-like dopamine receptor agonist SKF-38393 markedly increased cAMP accumulation, this being most prominent at 5 min of exposure to the agonist (39%) and was progressively lost with the increase of exposure time to the agonist [10 min, 23% increase; 25 min, 2% increase (Fig. 4B)]. The maximal inhibition on NHE activity (Fig. 5A) and the maximal stimulation of cAMP accumulation (Fig. 5B) obtained after 5 min exposure to SKF-38393 were both concentration-dependent effects and prevented by the selective D₁-like dopamine receptor antagonist SKF-83566 (Fig. 6, A and B).

The recovery of pHᵢ was clearly an Na⁺/H⁺-dependent process in IEC-6 cells and treatment for 5 min with SKF-38393 (1 μM) markedly attenuated the Na⁺/H⁺-dependent recovery of pHᵢ. As shown in Fig. 7, the maximal rate at which the pHᵢ recovery occurred (in pH units/s) in IEC-6 cells treated with 1 μM SKF-38393 (0.0025 ± 0.0003) was lower (P < 0.05) than that in vehicle-treated cells (0.0038 ± 0.0003), with similar Km values (in mM) for Na⁺ (vehicle, 14 ± 5; SKF-38393, 10 ± 5). The finding that Na⁺ removal reduced ~90% of the recovery of pHᵢ excludes the contribution of the H⁺-ATPase and clearly demonstrates a specific contribution of NHE activity during the alkalinization process after the Na⁺ removal.

To clarify which isoform of NHE is under control of dopamine D₁-like receptor stimulation, specific inhibitors to NHE1 and NHE3 were used. As shown in Fig. 8, the SKF-38393-mediated inhibitory effect on the exchanger activity was blocked by the specific NHE1 inhibitor HOE-642 (Fig. 8A) but not by the NHE3 inhibitor S-3226 (Fig. 8B), suggesting that dopamine D₁-like receptors attenuate the Na⁺/H⁺-dependent recovery of pHᵢ through inhibition of NHE1.

Decreased sensitivity to D₁-like stimulation may be explained by regulatory mechanisms of dopamine D₁-like receptor function and phosphorylation may be involved. Both PKA and GRKs can participate in this process. Moreover, stimulation of IEC-6 cells with SKF-38393 produced a concentration-dependent increase of cAMP intracellular content (Fig. 5B). As shown in Fig. 9A, overnight pretreatment with the nonselective GRK inhibitor heparin (1 μM) prevented the loss of D₁-like dopamine receptor-mediated inhibition of NHE activity after 25 min exposure to 1 μM SKF-38393 (from 4 ± 3% to 26 ± 4% reduction). This cell line expresses GRK4, GRK6A, and GRK6B as confirmed by immunoblotting studies (Fig. 3) and overnight treatment of IEC-6 cells with the anti-GRK4–6 antibody also prevented the loss of the stimulatory effect on cAMP accumulation after 25-min exposure to SKF-38393 (Fig. 10). To evaluate whether inhibition of
NHE activity after 25-min exposure to SKF-38393 was a cAMP-dependent effect, IEC-6 cells treated overnight with the anti-GRK4–6 antibody were treated with SKF-38393 (1 μM) for 25 min in the absence and the presence of PKA inhibitor H-89 (10 μM). As shown in Fig. 11, H-89 prevented the inhibitory effect SKF-38393, suggesting this is a cAMP-PKA-mediated event.

**DISCUSSION**

The results presented here show that rat IEC-6 intestinal epithelial cells are endowed with dopamine D₁ receptors, the stimulation of which results in inhibition of NHE activity and AC stimulation. The use of selective inhibitors of NHE1 and NHE3 isoforms suggests that dopamine D₁-like receptors attenuate the Na⁺-dependent recovery of pHᵢ through inhibition of NHE1. These effects, however, rapidly diminish, but were perpetuated by treatment of cells with the nonspecific GRK inhibitor heparin or the anti-GRK4–6 antibody. The dopamine D₁-like receptor-induced NHE inhibition is a cAMP-mediated event, but PKA is probably not to be involved in receptor desensitization.

GRKs can be regulated by desensitization, and it is generally accepted that desensitization involves receptor phosphorylation of which results in inhibition of NHE activity and AC stimulation. The use of selective inhibitors of NHE1 and NHE3 isoforms suggests that dopamine D₁-like receptors attenuate the Na⁺-dependent recovery of pHᵢ through inhibition of NHE1. These effects, however, rapidly diminish, but were perpetuated by treatment of cells with the nonspecific GRK inhibitor heparin or the anti-GRK4–6 antibody. The dopamine D₁-like receptor-induced NHE inhibition is a cAMP-mediated event, but PKA is probably not to be involved in receptor desensitization.

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tion (7, 8). PKA- and PKC-mediated phosphorylation uncouple the receptors from their respective G protein, whereas GRK-mediated desensitization is initiated by agonist-induced conformational changes in the receptor that upon phosphorylation becomes a substrate of arrestins, a family of proteins that participates in internalization of the receptors (25). The participation of PKA in dopamine D1 receptor desensitization has been previously reported, although there are some discrepancies. Some authors have shown that intracellular inhibitors of PKA (36) or elimination of potential phosphorylation sites in the receptors via site-directed mutagenesis (13) could attenuate D1 receptor desensitization and that PKA phosphorylation was involved in D1 receptor trafficking (19). In contrast, other authors (2, 16) provided data supporting the view that PKA does not have a relevant role in dopamine D1 receptor homologous desensitization. The present study shows that dopamine D1 receptors in the intestinal epithelial cell line IEC-6 rapidly desensitize to D1-like stimulation and increases in cAMP or activation of PKA are not responsible for desensitization. In fact, increases in intracellular cAMP after stimulation with D1-like agonist SKF-38393 attained its maximum at 5-min exposure to the agonist and rapidly dissipated. On the other hand, exposure of cells for 25 min with the membrane-permeable cAMP analog dibutyryl-cAMP or with the AC activator forskolin effectively inhibited NHE activity in IEC-6 cells.

GRKs are serine-threonine protein kinases that are implicated in homologous desensitization of a variety of G protein-coupled receptors (1, 5, 9, 29). Because GRKs are known to be involved in homologous desensitization of a variety of G protein-coupled receptors (1, 5, 9, 29). Because GRKs are known to be involved in homologous desensitization of a variety of G protein-coupled receptors, results in rapid desensitization, as evidenced by decreases in NHE inhibition and AC activity, is suggested to occur as a result of GRK-mediated phosphorylation of dopamine D1-like receptors, namely those belonging to the GRK4 and GRK6 subgroup.

REFERENCES


