Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na\(^+\)-K\(^+\)-ATPase activity

PEDRO GOMES AND P. SOARES-DA-SILVA

Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

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and phorbol esters on Na\(^+-K^+\)-ATPase activity were reported (12, 13, 42, 46) to occur through one of two mechanisms. The first involves direct phosphorylation of the Na\(^+\) pump at Ser-23 of the \(\alpha\)-subunit, leading to endocytosis of pumps (12, 13). The second mechanism involves activation of PL\(_A2\) and arachidonic acid metabolism (42, 46), but this was found not to be the case in well-oxygenated renal tubules (21). This led to the suggestion that phorbol esters inhibit proximal tubule Na\(^+-K^+\)-ATPase activity as a result of poor metabolic status that triggers cell-protective mechanisms (22, 53). Recently, a correlation has been reported (34) between PKA-dependent phosphorylation of the Na\(^+\) pump and activation of ouabain-sensitive Rb\(^+\) uptake and Na\(^+-K^+\)-ATPase activity in oxygenated, but not hypoxic, conditions. In addition, similar to PKA, PKC's effects on Na\(^+-K^+\)-ATPase activity are dependent on Ca\(^{2+}\) concentration (10). On the other hand, PKC-dependent activation of the Na\(^+-K^+\)-ATPase in the proximal nephron appears to be secondary to an increase in Na\(^+\) influx possibly via the Na\(^+/H^+\) exchanger (6), and the activation seems to be an oxygen-dependent process (21). Although direct phosphorylation of the Na\(^+-K^+\)-ATPase by PKA and/or PKC is an attractive and simple mechanism, the regulation of the Na\(^+\) pump is still a controversial issue. It is apparent, however, in a review of the literature (22, 53) that some of the difficulties arise because some of the experimental models used may not represent the ideal conditions for the assay of Na\(^+-K^+\)-ATPase activity (i.e., hypoxic conditions, low Ca\(^{2+}\), changes in Na\(^+\) influx via the Na\(^+/H^+\) exchanger).

The present study investigated the molecular events set into motion by stimulation of D\(_1\)-like receptors downstream of Na\(^+-K^+\)-ATPase in polarized opossum kidney (OK) cells, while using an electrophysiological model that requires continuous oxygenation of the medium in contact with both the apical and basolateral cell sides. The OK cell line is frequently used as a model of the tubular proximal epithelium and expresses characteristics useful for the study of the renal dopaminergic system (3, 4, 13, 26–28, 40, 43, 55, 56). The results reported here indicate that inhibition of Na\(^+-K^+\)-ATPase activity after stimulation of D\(_1\)-like receptors involves both the AC-PKA and the PLC-PKC systems. D\(_1\)-like receptors in OK cells are coupled to G\(_{4,4}\) but not G\(_{q/11}\) proteins, and the chain of molecular events begins with activation of the AC-PKA system followed by activation of the PLC-PKC system, PLC being an effector protein for PKA.

**METHODS**

**Cell culture.** OK cells, an established cell line derived from the kidney of a female American opossum, were obtained from the American Type Culture Collection (ATCC 1840 CRL, Rockville, MD) and maintained in a humidified atmosphere of 5% CO\(_2\)-95% air at 37°C. OK cells were grown in MEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin G, 0.25 µg/ml amphotericin B, 100 µg/ml streptomycin (Sigma), and 25 mM HEPES (Sigma). For subculturing, the cells were dissociated with 0.05% trypsin-EDTA (Sigma), split 1:5, and subcultured in petri dishes with a 21-cm\(^2\) growth area or six-well culture clusters (Costar, Badhoevedorp, The Netherlands). For electrophysiological studies, the cells were seeded onto polycarbonate filter supports (Snapwell, Costar) at a density of 13,000 cells/well. The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of initial seeding. For 24 h before each experiment, the cell medium was free of fetal bovine serum. Experiments were generally performed 2 days after cells reached confluence and 4 days after initial seeding, and each square centimeter contained ~100 µg of cell protein. In some experiments, cells were treated overnight on the apical cell side with agents known to interfere with signal transducing pathways, namely G proteins, such as cholera toxin (Sigma) and specific antibodies raised against G\(_\alpha\) or G\(_{q/11}\) proteins (Calbiochem, San Diego, CA). To minimize difficulties in antibodies entering the cell, anti-G\(_\alpha\) and anti-G\(_{q/11}\) antibodies (1:500) were prepared in the presence of lipofectin (1%, vol/vol; GIBCO-BRL, Grand Island, NY) and fetal bovine serum-free culture medium. On the day of the experiment, culture medium containing the test agents was removed, and the cells were washed with fresh medium and allowed to stabilize for at least 2 h before the start of acquisition of the electrophysiological parameters.

**Electrogenic ion transport in OK cells.** All transport experiments were conducted under short-circuit conditions. OK cells grown on polycarbonate filters (Snapwell, Costar) were mounted in Ussing chambers (window area, 1 cm\(^2\)) equipped with water-jacketed gas lifts bathed on both sides with 10 ml of Krebs-Hensleit solution, gassed with 95% O\(_2\) and 5% CO\(_2\), and maintained at 37°C. The standard composition of the apical and basolateral bathing Krebs-Hensleit solution was (in mM) 118 NaCl, 4.7 KCl, 25 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), and 1.2 MgSO\(_4\); pH was adjusted to 7.4 after gassing with 5% CO\(_2\) and 95% O\(_2\). The apical bathing Krebs-Hensleit solution contained mannitol (10 mM) instead of glucose (10 mM) to avoid entry of apical Na\(^+\) through the Na\(^+\)-dependent glucose transporter. The experimental design also required modification of the bathing solution compositions for specific experiments, and these changes are indicated below. After 5-min stabilization, monolayers were continuously voltage clamped to zero potential differences by application of external current, with compensation for fluid resistance, by means of an automatic voltage current clamp (DVC 1000, World Precision Instruments, Sarasota, FL). Transepithelial resistance (Ω-cm\(^2\)) was determined by altering the membrane potential stepwise (±3 mV) and applying the ohmic relationship. Cells were allowed to stabilize for 25 min before permeabilization with amphotericin B; this period was also used for exposure of cells to the relevant drug treatments. The voltage/current clamp unit was connected to a personal computer via a BIOPAC MP1000 data-acquisition system (Goleta, CA). Data analysis was performed using AcqKnowledge 2.0 software (BIOPAC Systems).

**Na\(^+-K^+\)-ATPase activity.** The effect of dopamine and D\(_1\)-like receptor agonists on Na\(^+-K^+\)-ATPase activity was examined in monolayers mounted in Ussing chambers bathed with the standard Krebs-Hensleit solution, so that the final bath Na\(^+\) concentration was 145 mM on both sides of the monolayers. The apical membrane was then permeabilized by addition of amphotericin B to the apical bathing solution. Under short-circuit conditions, the resulting current is due to the transport of Na\(^+\) across the basolateral membrane by the Na\(^+-K^+\)-ATPase (15, 27, 55). This experimental model allows the entry of apical Na\(^+\) and leads to inhibition of the Na\(^+/H^+\) exchanger (27). The concentration-response relationship of the short-circuit current (\(I_{sc}\)) for bath Na\(^+\) was evaluated by...
initially bathing the apical side of the monolayers mounted in Ussing chambers with Na+-free Krebs-Henseleit solution (NaCl replaced with choline chloride and NaHCO3 replaced with choline bicarbonate). Amphotericin B was then administered to the apical bathing solution, and Iw was continuously recorded. Thereafter, the Na+ concentration was incrementally increased by removing bathing medium from the apical side of the monolayers and replacing it with equal volumes of normal Krebs-Henseleit solution. Thus bath Na+ concentration was gradually increased from 0 to 143 mM without affecting the concentrations of other ions. In some experiments, amphotericin B was applied from the basolateral cell side. All test drugs were applied to both the apical and basolateral cell sides, with the following exceptions: amiloride (apical only), ouabain (basolateral only), DIDS (basolateral only), barium chloride (basolateral only), and SKF-83566 (apical only). SKF-38393 in some experiments (indicated in text) was applied to the basolateral cell side; in all remaining experiments, SKF-38393 was applied to the apical cell side on.

**cAMP measurement.** Intracellular cAMP was determined with an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI), as previously described (27). OK cells were preincubated for 15 min at 37°C in Hanks’ medium (medium composition in mM: 137 NaCl, 5 KCl, 0.8 M MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 0.25 CaCl2, 1 MgCl2, 0.15 Tris-HCl, and 1 sodium butyrate, pH 7.4), containing 100 μM IBMX, a phosphodiesterase inhibitor. Cells were then incubated for 15 min with test compounds. In some experiments, cells were treated overnight from the apical cell side in the presence of anti-Gα and anti-Gq/11 antibodies (1:500) prepared in 1% vol/vol, as described in Cell culture. At the end of the experiment, the reaction was stopped on ice, the incubation medium was discarded, and the cell monolayer was added with 0.1 M HCl. Aliquots were then taken for the measurement of intracellular cAMP content.

**PLC activity.** OK cells grown in six-well culture clusters were incubated for 15 min at 37°C with test compounds in Hanks’ medium. Washing the cells three times with ice-cold Hanks’ medium terminated the incubations. Subsequently, the cells were lysed by adding lysis buffer containing (in mM) 20 Tris-HCl, pH 7.4, 2 EDTA, 2 phenylmethylsulfonyl fluoride (PMSF), 25 sodium pyrophosphate, and 20 sodium fluoride and 10 μg/ml each leupeptin and aprotonin. Thereafter, the cells were centrifuged at 4,000 rpm for 20 min at 4°C, and the cytosol and membrane fractions were separated for the assay of PLC activity. The cytosol and membranes were assayed for PLC activity using the Amplex Red phosphatidylinositol-specific PLC assay kit (Molecular Probes, Eugene, OR), using a Spectramax Gemini dual-scanning fluorescence microplate reader (Molecular Devices). Briefly, PLC was monitored indirectly using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H2O2. Assays were performed in 96-well plates, with 200 μl reaction volume. First, PLC converts the phosphatidylinositol (lecithin) substrate to form phosphocholine and diacylglycerol. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H2O2. Finally, H2O2, in the presence of horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry, to generate the highly fluorescent product resorufin. PLC activity was expressed as relative fluorescence units per milligram of protein.

**Intracellular Ca2+ measurement.** Intracellular Ca2+ was measured as previously described (27). At day 4 after seeding, the glass coverslips were incubated at 37°C for 40 min with 5 μM of the Ca2+-dependent fluorescent indicator fura-2. Coverslips were then washed twice with prewarmed dye-free modified Krebs buffer [buffer composition in mM: 140 NaCl, 5.4 KCl, 2.5 CaCl2, 1.2 MgSO4, 0.3 NaH2PO4, 0.3 KH2PO4, 10 HEPES, and 5 glucose (pH to 7.4 with Tris base)] before initiation of the fluorescence recordings. Cells were mounted diagonally in a 1 × 1-cm acrylic fluorometric cuvette and placed in the sample compartment of a FluoroMax-2 spectrofluorometer (Jobin Yvon-SPEX, Edison, NJ). The cuvette volume of 3 ml was constantly stirred and perfused at 5 ml/min with modified Krebs buffer prewarmed to 37°C. Under these conditions, the cuvette medium was replaced within ~150 s. After 5 min, fluorescence was measured every 5 s alternating between 340- and 380-nm excitation (slit size, 2 nm) at 510-nm emission (slit size, 5 nm). The ratio of intracellular fura 2 fluorescence at 340 and 380 nm was an index of intracellular Ca2+.

**Western blotting.** Membranes were prepared from OK cells and the kidney outer cortex of male Wistar rats (Harlan). Both samples were added to 25 mM HEPES, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM PMSF, homogenized (Dixum homogenizer, Heidolph) and centrifuged for 10 min at 500 g. The supernatant was then centrifuged at 50,000 g for 20 min, and the membrane pellet was resuspended in HEPES buffer. Membrane proteins (30–35 μg) were solubilized in Laemmi buffer and resolved by SDS-PAGE (10% acrylamide) together with the molecular weight marker Benchmark prestained protein ladder (Life Technologies). The resolved proteins were electrochemically transferred onto nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech). The blots were blocked with 5% skim milk in Tween-Tris-buffered saline (0.1% Tween 20, 154 mM NaCl, and 100 mM Tris, pH 7.5) containing 0.02% NaN3. The blots were incubated with anti-Gα or anti-Gq/11 subunit, COOH-terminal or anti-Gqα11 subunit, COOH-terminal antibodies (1:1,000 dilution, Calbiochem) for 1 h at room temperature. The antibodies bound to nitrocellulose were detected by incubation with a 1:1,000 dilution of an anti-rabbit IgG-alkaline phosphatase antibody (Roche, Basel, Switzerland) and then incubation in 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenylothiazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche).

**Downregulation studies.** PKA and PKC downregulation was performed by overnight exposure to cAMP (100 μM) or phorbol 12,13-dibutyrate (PDBu; 100 nM), respectively, as previously described (49, 54).

**Protein assay.** The protein content of monolayers of OK cells was determined by the Bradford method (8), with human serum albumin as a standard.

**Data analysis.** Arithmetic means are given with SE or geometric means with 95% confidence values. Statistical analysis was done with one-way ANOVA followed by a Newman-Keuls test for multiple comparisons. P < 0.05 was assumed to denote significant difference.

**Drugs.** Amphotericin B, arachidonic acid, chelerythrine chloride, chelerythrine chloride, dicubryl cAMP (DBcAMP), DIDS, etoxyseroxurin, forskolin, H-89, IBMX, okadaic acid, ouabain, PDBu, 4α-phorbol 12,13-didecanoate, trypsin, and U-73122-LY-294002, PD-098059, and wortmannin were purchased from Sigma. (±)-SKF-83566 hydrochloride and (±)-SKF-38393 hydrochloride were obtained from Research Biochemicals (Natick, MA). Fura 2 was obtained from Molecular Probes.

**RESULTS**

Under conditions of 143 mM Na+ in the extracellular medium, the addition of amphotericin B to the apical...
cell side induced an increase in $I_{sc}$; this effect was dependent on the concentration used (Fig. 1A and Table 1). The maximum effect was attained at 3 μg/ml amphotericin B; the effect of 5 μg/ml amphotericin B (data not shown) was similar to that obtained at a lower concentration (3 μg/ml). Thus, in all subsequent experiments, the apical membrane was permeabilized with 1 μg/ml amphotericin B, to increase the Na⁺ delivered to Na⁺-K⁺-ATPase to the half-maximal saturating level. Under these conditions, the amphotericin B (1 μg/ml)-induced increase in $I_{sc}$ was markedly ($P < 0.05$) attenuated (92% reduction) by removing Na⁺ from the solution bathing the apical cell border (Fig. 1B). Similarly, removal of K⁺ from the solution bathing the basolateral cell side (substitution by cesium chloride) markedly attenuated (87% reduction) the amphotericin B (1 μg/ml)-induced increase in $I_{sc}$ (Fig. 1B). As shown in Fig. 1B, the increase in $I_{sc}$ induced by amphotericin B applied to the apical cell side was not affected by the K⁺ channel blocker barium chloride (1 mM) and the Na⁺-HCO₃⁻ cotransport inhibitor DIDS (200 μM). In additional experiments aimed at determining the cell border on which the driving force that generates the amphotericin B-induced current is localized, the ionophore was applied to either the apical or basolateral cell side. As shown in Fig. 2, the increase in $I_{sc}$ observed with the addition of amphotericin B to the apical cell side was 6.6-fold that observed when amphotericin B was applied to the basolateral cell side. The increase in $I_{sc}$ elicited by apical amphotericin B was markedly ($P < 0.05$) attenuated (78% reduction) by ouabain applied to the basolateral cell side but insensitive to amiloride applied to the apical cell side (Fig. 2A). In contrast, the increase in $I_{sc}$ elicited by basolateral amphotericin B was significantly different from corresponding control value.

Table 1. Effect of increasing concentrations of amphotericin B on $I_{sc}$ in OK cells

<table>
<thead>
<tr>
<th>Amphotericin B, μg/ml</th>
<th>$I_{sc}$, μA/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.2 ± 1.6</td>
</tr>
<tr>
<td>0.3</td>
<td>10.2 ± 2.2</td>
</tr>
<tr>
<td>0.6</td>
<td>22.7 ± 1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>43.0 ± 1.9</td>
</tr>
<tr>
<td>3.0</td>
<td>70.7 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. $I_{sc}$, short-circuit current; OK, opossum kidney.
significantly ($P < 0.05$) attenuated (57% reduction) by amiloride applied to the apical cell side but insensitive to ouabain applied to the basolateral cell side (Fig. 2A). Taken together, these results suggest that increases in $I_{sc}$ elicited by apical amphotericin B reflect increases in the activity of Na$^+-$K$^+$.ATPase located in the basolateral membrane.

Pretreatment of OK cell monolayers with dopamine (1 μM) applied to the basolateral cell border failed to affect the amphotericin B-induced increase in $I_{sc}$ (Table 2). In contrast, when dopamine (1 μM) was applied to the apical cell border, a significant decrease (29 ± 5% reduction) in the amphotericin B-induced increase in $I_{sc}$ was observed, this being prevented by the D$_1$-like receptor antagonist SKF-83566 (1 μM) (Table 2). The selective D$_1$-like receptor agonist SKF-38393 (30 to 1,000 nM; apical application) was also found to attenuate, in a concentration-dependent manner, the amphotericin B-induced increase in $I_{sc}$ (Fig. 3). The relationship between the amphotericin B-induced increase in $I_{sc}$ and the concentration of extracellular Na$^+$ showed a Michaelis-Menten constant ($K_m$) of 37.6 ± 10.2 mM and a $V_{max}$ of 49.0 ± 3.5 μA/cm$^2$ in control monolayers. However, in the presence of SKF-38393 (1 μM), $V_{max}$ was significantly reduced to 31.4 ± 1.4 μA/cm$^2$ without changes in $K_m$ values (39.2 ± 5.0 mM) (Fig. 4). Altogether, these results suggest that stimulation of D$_1$-like receptors does not alter the affinity of Na$^+-$K$^+$.ATPase for Na$^+$; instead, the stimulation reduces the rate at which Na$^+$ pump units extruded intracellular Na$^+$. The apparent affinity of Na$^+-$K$^+$.ATPase for Na$^+$ in OK cells was in the same range of magnitude as that described previously in colonic epithelial cells (20 mM Na$^+$) using an identical electrophysiological methodology (15).

Next, we evaluated the involvement of G proteins in the regulation of Na$^+-$K$^+$.ATPase in OK cells. Cholera toxin was used to maximally activate the GTP-binding protein (G protein) G$\alpha$ and uncouple the D$_1$-like receptor from G$\alpha$ (37). Overnight treatment of OK cells with cholina toxin (500 ng/ml) abolished the effect of SKF-38393 (300 nM) on the amphotericin B-induced increase in $I_{sc}$ (Table 2). This was accompanied by increases in both the basal levels of cAMP and the forskolin (3 μM)-stimulated accumulation of cAMP (Table 3). On the other hand, SKF-38393 (300 nM) stimulated cAMP production, this being prevented by the specific D$_1$-like receptor antagonist SKF-83566 (1 μM) (Table 3).

Previous studies (32) have demonstrated that second messenger pathways thought to be involved in D$_1$-like receptor-mediated inhibition of Na$^+-$K$^+$.ATPase include stimulation of PKA or PKC pathways. To evaluate whether this was the case in OK cells using electrophysiological techniques under in vivo experimental conditions, we examined the effects of DBcAMP, a direct activator of PKA, and PDBu, a potent activator of PKC. Treatment of OK cells with increasing concentrations of DBcAMP (100–500 μM) (Fig. 5A) and PDBu

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**Table 2. Changes in amphotericin B-induced increase in $I_{sc}$ in OK cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{sc}$ (μA/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 6.6</td>
</tr>
<tr>
<td>Dopamine (basal)</td>
<td>92.4 ± 4.3</td>
</tr>
<tr>
<td>Dopamine (apical)</td>
<td>71.4 ± 4.5</td>
</tr>
<tr>
<td>+ SKF-83566 (apical)</td>
<td>106.4 ± 5.9†</td>
</tr>
<tr>
<td>SKF-38393</td>
<td>71.2 ± 5.2†</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>100.0 ± 5.4</td>
</tr>
<tr>
<td>+ SKF-38393</td>
<td>95.6 ± 5.1†</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>100.0 ± 7.5</td>
</tr>
<tr>
<td>+ SKF-38393</td>
<td>70.4 ± 6.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE given as %control for amphotericin B-induced increases in $I_{sc}$ (absolute level was 36 ± 2.4 μA/cm$^2$; $n = 10$). $I_{sc}$ changes were measured under control conditions and in the presence of dopamine (1 μM; applied to the basal or apical cell border), dopamine + SKF-83566 (both applied to the apical cell border at 1 μM), or SKF-38393 (applied to the apical cell border at 0.3 μM). In some experiments, cells were treated overnight with cholera toxin (500 ng/ml) or pertussis toxin (100 ng/ml). *$P < 0.05$, significantly different from control values; †$P < 0.05$, significantly different from values for dopamine or SKF-38393 alone.

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**Table 3. Changes in Na$^+$ dependent $I_{sc}$ in OK cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{sc}$ (μA/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.0 ± 2.4</td>
</tr>
<tr>
<td>Dopamine (basal)</td>
<td>31.4 ± 1.4</td>
</tr>
<tr>
<td>+ SKF-38393</td>
<td>49.0 ± 2.4</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>29.5 ± 1.5†</td>
</tr>
<tr>
<td>+ SKF-38393</td>
<td>25.0 ± 1.5†</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>25.0 ± 1.5†</td>
</tr>
<tr>
<td>+ SKF-38393</td>
<td>25.0 ± 1.5†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 2–11 experiments/group. *$P < 0.05$, significantly different from control value.
ever, PDBu was still able to inhibit the amphotericin B-induced increase in $I_{sc}$ (Fig. 7). The ability of PDBu to inhibit the amphotericin B-induced increase in $I_{sc}$ in PKA downregulation and the failure of SKF-38393 and DBcAMP to reduce the amphotericin B-induced increase in $I_{sc}$ in PKC downregulation suggest that PKA may be activated before PKC activation. One possible sequence of events might be the activation of PLC by PKA, before activation of PKC. Therefore, we next evaluated the involvement of PLC in the inhibitory effects of the D$_1$-like receptor agonist SKF-38393, DBcAMP, and PDBu. We tested the effect of U-73122, a PLC inhibitor (7), on the effect of SKF-38393, DBcAMP, and PDBu. As shown in Fig. 8, U-73122 (3 $\mu$M) was able to prevent the inhibitory effects of both DBcAMP (200 $\mu$M) and SKF-38393 (300 nM) on the amphotericin B-induced increase in $I_{sc}$ (Fig. 8). However, the PLC inhibitor U-73122 (3 $\mu$M) did not affect the inhibitory effect of 100 nM PDBu (Fig. 8). Similarly, U-73122 (3 $\mu$M) failed to alter stimulation of cAMP production by forskolin (3 $\mu$M) (Table 3). This suggests that PLC activation may occur downstream of AC activation. To confirm this view, we also performed

Table 3. Changes in cAMP levels in OK cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>152 ± 12*</td>
</tr>
<tr>
<td>Forskolin</td>
<td>186 ± 5*</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>PDBu</td>
<td>131 ± 19‡</td>
</tr>
<tr>
<td>SKF-38393</td>
<td>217 ± 24*</td>
</tr>
<tr>
<td>+ SKF-38366 (1 $\mu$M)</td>
<td>131 ± 19‡</td>
</tr>
<tr>
<td>+ PDBu</td>
<td>221 ± 20*</td>
</tr>
<tr>
<td>+ Thapsigargin</td>
<td>164 ± 19*</td>
</tr>
</tbody>
</table>

Values are means ± SE given as %control for cAMP accumulation (absolute level was 25 ± 4 pmol/mg protein; n = 18). Changes in cAMP levels measured under control conditions and after exposure to cholera toxin (500 ng/ml; overnight exposure), forskolin (3 $\mu$M), thapsigargin (1 $\mu$M), and phorbol 12,13-dibutyrate (PDBu; 1 $\mu$M). *P < 0.05, significantly different from corresponding control values; †P < 0.05, significantly different from values for forskolin alone; ‡P < 0.05, significantly different from values for agonist alone.

(10–1,000 nM) (Fig. 5B), applied from both cell sides, effectively reduced the amphotericin B-induced increase in $I_{sc}$. The inactive phorbol ester 4α- phorbol 12,13-didecanoate (1 $\mu$M) did not affect the changes induced by amphotericin B (data not shown). To confirm the involvement of PKA and PKC pathways, we used selective antagonists of PKA (H-89) and PKC (chelerythrine). As shown in Fig. 6A, H-89 (10 $\mu$M) antagonized the inhibitory effects of both DBcAMP (200 $\mu$M) and SKF-38393 (300 nM). Similarly, chelerythrine (1 $\mu$M) antagonized the effects of both PDBu (100 nM) and SKF-38393 (300 nM) (Fig. 6B). These results suggest that stimulation of D$_1$-like receptors may lead to simultaneous activation of both PKA and PKC transduction pathways with a common point in the cascade of events, since either H-89 or chelerythrine completely prevented the effects of SKF-38393. To confirm the involvement of both PKA and PKC in the inhibition of Na$^+$-K$^+$-ATPase evoked by D$_1$-like receptor stimulation and clarify the sequence of events in more detail, we performed complementary studies involving downregulation of PKA and PKC. To promote PKA downregulation, we incubated OK cells overnight (~16–20 h) in the presence of DBcAMP (100 $\mu$M). Under these experimental conditions, the effects of both DBcAMP and SKF-38393 were abolished; however, PDBu was still able to inhibit the amphotericin B-induced increase in $I_{sc}$ (Fig. 7). In the application of a similar strategy to downregulate PKC activity, cells were incubated overnight (~16–20 h) in the presence of PDBu (100 nM). Under these conditions, PDBu, DBcAMP, and SKF-38393 had no effect on the amphotericin B-induced increase in $I_{sc}$ (Fig. 7).
complementary studies on PLC activity in OK cells. As shown in Fig. 9, DbcAMP (500 μM), but not PDBu (200 nM), increased cytosolic and membrane PLC activity in OK cells.

The signaling pathways linked to D1-like receptors include the coupling of Gsα and Gq11α proteins, respectively (18, 20, 57). We therefore felt it worthwhile to examine the presence and involvement of Gsα and Gq11α proteins in the events after stimulation of D1-like receptors with SKF-38393. The presence of Gsα and Gq11α proteins in OK cells was evaluated by Western blotting with specific antibodies from rabbit raised against the synthetic decapeptide of the COOH terminal of Gsα and Gq11α proteins. As shown in Fig. 10, these antibodies recognized the presence of both Gsα and Gq11α proteins in OK cells. For comparison, the rat kidney cortex was also evaluated for the presence of Gsα and Gq11α proteins. The anti-Gsα protein antibody recognized a single band of ~40 kDa in OK cells and a major band of ~40 kDa and a minor band of ~45 kDa in rat renal cortex. The anti-Gq11α protein antibody recognized a single band of ~36 kDa in both OK cells and rat renal cortex. The size of the α-subunits identified in this study is comparable to the size reported in other tissues (29). To evaluate the involvement of Gsα and Gq11α proteins in the inhibition of Na+-K+-ATPase evoked by D1-like receptor stimulation, further studies were performed in cells treated overnight (~16–20 h) with antibodies raised against rat Gsα and Gq11α proteins. As shown in Fig. 11, the inhibitory effect of SKF-38393 on the amphotericin B-induced increase in Isc was abolished in cells treated with the anti-Gsα antibody, but not in cells treated with the anti-Gq11α antibody. These results agree with the view that D1-like receptors in OK cells are coupled to AC via a Gsα-type of G protein and subsequent activation of PLC-PKC systems does not involve a Gq11α-type of G protein but most likely results from activation of PLC by PKA. As
shown in Table 3, the dopamine-induced increase in cAMP was abolished in cells treated with the anti-G\(_{\alpha}\) antibody. The dopamine-induced increase in cAMP was also completely prevented by pretreatment with the selective D\(_1\)-like receptor antagonist SKF-83566 (10 \(\mu\)M).

Other possible interactions between PKA and PKC pathways and common intracellular events may involve the regulation of AC. Certain AC in some cells are stimulated by Ca\(^{2+}\)/calmodulin, while others are inhibited by Ca\(^{2+}\) (51). Another mechanism by which AC activity can be regulated is PKC phosphorylation. The AC1, AC2, and AC3 isoforms are significantly stimulated by PKC activation; in contrast, the AC4, AC5, and AC6 isoforms are only modestly stimulated (51). For these reasons, we decided to evaluate the relationship between Ca\(^{2+}\), PKC activation, and cAMP production in the OK cell line. PKC activation with PDBu (300 nM) increased basal cAMP levels by 30\%, and forskolin (3 \(\mu\)M) stimulated levels by 75\% on average (Table 3). Treatment of the cells with thapsigargin (1 \(\mu\)M), the endosomal Ca\(^{2+}\)-ATPase inhibitor (38), evoked an immediate increase in intracellular Ca\(^{2+}\) (Fig. 12) but failed to alter both basal and for-
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Okadaic acid (50 nM) significantly enhanced the inhibitory effect of 30 nM SKF-38393 (Table 4).

Dopamine has also been shown to inhibit renal tubular Na⁺-K⁺-ATPase activity via the cytochrome P-450-monoxygenase pathway of the arachidonic acid cascade (47). Arachidonic acid (10–1,000 nM) produced no effect on changes in the amphotericin B-induced increase in \( I_{sc} \) (from 47.2 ± 3.2 to 46.3 ± 4.2 \( \mu A/cm^2 \), \( n = 3–7 \)). Ethoxyresorufin (100 nM), a specific inhibitor of the cytochrome P-450 pathway (9), failed to prevent the inhibitory effect of SKF-38393 (300 nM) on the amphotericin B-induced increase in \( I_{sc} \) (Table 4). Other studies (14) have also reported the involvement of phosphatidylinositol 3-kinase (PI3-kinase) during dopamine-mediated inhibition of Na⁺-K⁺-ATPase activity. Therefore it was decided to evaluate the effects of two inhibitors of PI3-kinase, wortmannin and LY-294002. As shown in Table 4, both wortmannin (100 nM) and LY-294002 (25 μM) failed to prevent the inhibitory effect of SKF-38393 upon amphotericin B-induced increase in \( I_{sc} \). The mitogen-activated PK inhibitor PD-098059 (10 μM) also failed to prevent the inhibitory effect of SKF-38393 (Table 4).

**DISCUSSION**

In the present study, while using the pore-forming antibiotic amphotericin B to permeabilize the apical membrane, we were able to isolate Na⁺ currents and assess the effects of dopamine or D₁-like receptor agonists on the basolateral membrane Na⁺-K⁺-ATPase activity, in intact OK cell monolayers. The polarized response to SKF-38393 (only when applied to the apical cell side) suggests that D₁-like receptors may be absent from the basolateral membranes or not accessible to the agonist. In the rat, D₁-like receptors have been described (18) in both the brush-border and basolateral membranes. More recently, in the rabbit cortical collecting duct, D₂-like receptors were found (45) to be present in the basolateral cell side only, whereas D₁-like receptors were exclusively distributed to the apical cell side. Kinetically, the D₁-like receptor-mediated decrease in Na⁺ transepithelial flux in OK cells was demonstrated to occur via a reduction in the \( V_{max} \) for Na⁺ without affecting the affinity of Na⁺-K⁺-ATPase for Na⁺. This finding is in agreement with previous studies (13) showing that inhibition of Na⁺-K⁺-ATPase activity in renal epithelial cells by activation of G protein-coupled receptors is mediated by phosphorylation of the catalytic \( \alpha \)-subunit followed by removal of active molecules from the plasma membrane. Phosphorylation may serve as the triggering signal in the removal process, but it does not affect Na⁺-K⁺-ATPase activity while it resides in the plasma membrane (12). The experimental conditions under which Na⁺-K⁺-ATPase activity was assessed require a constant oxygen supply to the preparation, and the concentration of Ca²⁺ in the medium was kept to a level expected to allow stimulation of the Na⁺ pump. Furthermore, under these experimental conditions, the addition of dopamine or PDBu was not accompanied by changes in intracellular Ca²⁺ concentration. Glucose was omitted from the apical, but not the basal, bathing solution to avoid entry of apical Na⁺ through the Na⁺-dependent glucose transporter. However, this is not expected to restrict metabolic requirements of OK cells. Accordingly, the PKC-mediated inhibition of Na⁺-K⁺-ATPase activity by dopamine, cAMP, and PDBu may not relate to hypoxic conditions, as a result of poor metabolic status or deficient Ca²⁺ availability. On the other hand, the data presented here shed some light on the possible role of the cAMP-PKA system on the dopamine-induced PKC-mediated inhibition of Na⁺-K⁺-ATPase activity.

The results from this study also provide direct evidence that D₁-like receptor-mediated inhibition of Na⁺-K⁺-ATPase activity involves a G protein of the \( G_\alpha \) class, but not of the \( G_{\alpha_{11}} \) class, positively coupled to AC. Transduction mechanisms set into motion during activation of D₁-like receptors in OK cells involve the activation of both PKA and PKC pathways in a single sequence of events with PKA activation occur-

**Table 4. Effects of SKF-38393 on changes in amphotericin B-induced increase in \( I_{sc} \)**

| Treatment                  | \( I_{sc} \)  
|----------------------------|--------------|
| Control                    | 100.0 ± 6.7  
| Okadaic acid              | 84.9 ± 4.6  
| SKF-38393 (30 nM)         | 84.0 ± 6.6  
| + Okadaic acid            | 68.3 ± 5.5  
| Control                    | 100.0 ± 4.5  
| SKF-38393 (300 nM)        | 71.2 ± 5.2  
| + Ethoxyresorufin         | 99.6 ± 4.3  
| + SKF-38393 (300 nM)      | 65.3 ± 4.8* |
| Wortmannin                 | 107.3 ± 1.2  
| + SKF-38393 (300 nM)      | 72.1 ± 1.7* |
| LY-294002                 | 94.4 ± 5.4  
| + SKF-38393 (300 nM)      | 75.8 ± 1.8* |
| PD-098059                 | 100.0 ± 1.9  
| + SKF-38393 (300 nM)      | 76.9 ± 4.5* |

Values are means ± SE (\( n = 3–7 \)) given as %control for amphotericin B (1 μg/ml)-induced increases in \( I_{sc} \) (absolute level was 47.2 ± 3.2 \( \mu A/cm^2 \), \( n = 7 \)). * indicates \( P < 0.05 \), significantly different from corresponding control value.
Dopamine regulation of renal Na+/K+-ATPase activity. The dopamine D1-like receptors have been shown (33, 58) to stimulate PLC-β1 via pertussis toxin-insensitive G proteins of the Gq family. However, our results contrast with the previous reports (33, 58), showing that the D1-like receptor was not positively coupled to the Gq11 protein. Activation of a phosphatidylinositol-specific PLC most likely occurs as a result of phosphorylation by PKA. This theory is based on experiments with antibodies raised against the carboxy terminal of Gsα and Gq11α subunits to block interactions of G proteins with D1-like receptors. The following observations support this conclusion: 1) Western blot analysis revealed the presence of both Gsα and Gq11α proteins in OK cells; 2) when cells were treated overnight with antibodies raised against rat Gsα and Gq11α proteins, the inhibitory effect of the D1-like receptor agonist SKF-38393 on the amphotericin B-induced increase in Isc was abolished in cells treated with the anti-Gsα antibody, but not in cells treated with the anti-Gq11α antibody; and 3) DBCAMP, but not PDBu, significantly stimulated PLC activity in both membrane and cytosol preparations from OK cells. Thus D1-like receptor agonists stimulate PLC and PKC activity in renal OK cells independent of Gq11α proteins. However, this is not the only type of interaction between the PKA and PKC transducing pathways in OK cells. Although this was not directly evidenced following D1-like receptor activation, PKC activation by PDBu was accompanied by increases in both basal and forskolin-stimulated cAMP levels. This indicates the presence of a positive coupling between PKC and PKA in OK cells that involves activation of AC. Whether this mechanism intensifies the effects of dopamine on the cell is not understood from the experiments presented here. However, considering the modest increase in cAMP accumulation produced by PDBu, it is unlikely that this significantly contributes to the interaction between PKA and PKC pathways in OK cells. On the other hand, increases in intracellular Ca2+ by thapsigargin failed to alter both basal and forskolin (3 μM)-stimulated cAMP levels. This would agree with the view that AC isoforms in OK cells may be of AC4, AC5, and AC6 types, considering their modest sensitivity to PDBu and insensitivity to increases in intracellular Ca2+. The failure of dopamine and PDBu to alter intracellular Ca2+ levels in OK cells also suggests that PKC-mediated inhibition of Na+/K+-ATPase activity in OK cells may be not associated with marked changes in intracellular Ca2+.

To our knowledge, this chain of events constitutes a new signaling pathway, downstream D1-like dopamine receptor activation leading to inhibition of Na+/K+-ATPase activity. Other studies (5) have reported on complex processes leading to inhibition of Na+/K+-ATPase activity, namely, the requirement of simultaneous activation of both D1-like and D2-like receptors. Although the OK cells are endowed with both D1-like and D2-like receptors, it is unlikely that D2-like receptors are involved in the generation of responses leading to direct inhibition of Na+/K+-ATPase activity. In fact,
SKF-38393 is a rather selective D₁-like receptor agonist, the effects of which have been shown, in the OK cell line, to be insensitive to the selective D₂-like receptor antagonist S-sulpiride (27). Another example in which the involvement of both PKA and PKC activation was observed downstream of dopamine receptor activation is that of LTK cells stably transfected with the rat D₁ receptor cDNA (59). Yu et al. (59) showed that the D₁-mediated stimulation of PLC occurred as a result of PKA activation via stimulation of PKC. This model contrasts with our proposal in OK cells (Fig. 13), the main arguments being the lack of involvement of the G₁₁α type of G protein and the finding that inhibition of PLC by U-73122 failed to prevent inhibition of Na⁺-K⁺-ATPase activity by PDBu. Dual coupling to AC and PLC has been reported (44) in OK cells for parathyroid hormone (PTH). However, it is likely that PTH signal transduction via a cAMP-dependent pathway does not involve stimulation of PLC (44). This has been also observed (17, 23, 24, 35) for other types of receptors, in different tissues, in which dual coupling to AC and PLC generally involves independent pathways. Recently, however, it has been shown (48) that a new PLC and Ca²⁺ signaling pathway was triggered by cAMP and mediated by a small GTPase of the Rap family. These events, resulting from stimulation of β₂-adrenoceptors in HEK-293 cells or the endogenous receptor for PGE₁ in N1E-115 neuroblastoma cells, were caused by cAMP elevation, but independent of PKA (48). This contrasts with our observation in OK cells that stimulation of the D₁-like receptor leads to AC-PKA activation followed by activation of PLC by PKA (Fig. 13).

PLA₂ is a potential third pathway by which the D₁-like receptor transduces its signal to Na⁺-K⁺-ATPase in the proximal tubules (41, 47). There is evidence suggesting this involves the PLA₂-arachidonic acid-20-HETE pathway (41, 47). 20-HETE is a metabolite of arachidonic acid, and PKC. The lack of involvement of the PLA₂-arachidonic acid-20-HETE pathway in OK cells may explain why PI3-kinase inhibitors fail to affect the response to D₁-like receptor stimulation. It is possible this different behavior may relate to differences between species (rat and opossum).

In conclusion, it is suggested that the D₁-mediated inhibition of Na⁺-K⁺-ATPase activity in OK cells sequentially involves the AC-PKA system and the PLC-PKC system. The results from this study provide direct evidence that inhibition of Na⁺-K⁺-ATPase activity by dopamine in OK cells involves the activation of D₁-like dopamine receptors and a G protein of the G₁₁α class positively coupled to AC. Transduction mechanisms set into motion during activation of D₁-like receptors in OK cells involve the activation of both PKA and PKC pathways in a single sequence of events, with PKA activation prior to PKC activation, which most likely includes activation of PLC by PKA.

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