Bimodal Acute Effects of $A_1$ Adenosine Receptor Activation on $Na^+/H^+$ Exchanger 3 in Opossum Kidney Cells

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Abstract. Regulation of renal apical $Na^+/H^+$ exchanger 3 (NHE3) activity by adenosine has been suggested to contribute to acute control of mammalian $Na^+$ homeostasis. The mechanism by which adenosine controls NHE3 activity in a renal cell line was examined. The adenosine analog, N6-cyclopentyladenosine (CPA) exerts a bimodal effect on NHE3: CPA concentrations $>10^{-8}$ M inactivate NHE3, whereas concentrations $<10^{-8}$ M stimulate NHE3 activity. Acute CPA-induced control of NHE3 was blocked by antagonists of $A_1$ adenosine receptors and inhibition of phospholipase C, pretreatment with BAPTA-AM (chelator of cellular calcium), and exposure to pertussis toxin. Stimulatory and to some extent also inhibitory CPA concentrations attenuated 8-bromo-cAMP and dopamine-mediated inhibition of NHE3. BAPTA eliminated the ability of a stimulatory dose of CPA to attenuate 8-bromo-cAMP–induced suppression of NHE3 activity. Upon inhibition of protein kinase C, CPA at an inhibitory dose provoked activation of NHE3, which is partially reverted by 8-bromo-cAMP and suppressed by pre-incubation with BAPTA-AM. Cytochalasin B, an actin-modifying agent, selectively prevented downregulation but did not affect upregulation of NHE3 activity by CPA. In conclusion, these observations demonstrate that (1) CPA modulates NHE3 activity by elevation of cellular Ca$^{2+}$ exerting a negative control on adenylate cyclase activity, (2) protein kinase C is the determining factor leading to CPA-induced downregulation of NHE3 activity, and (3) alterations of surface NHE3 abundance may contribute to $A_1$ adenosine receptor–dependent inhibition of NHE3 activity.

Adenosine locally released in tissues under conditions causing hypoxia, ischemia, or inflammation modulates many aspects of physiologic functions in mammals through binding to $A_1$, $A_2A$, $A_2B$, and/or $A_3$ adenosine receptors (1). Of the four adenosine receptors that have been cloned, $A_1$, $A_2A$, and $A_3$ adenosine receptors are known to participate in renal physiology (1–7). Regarding signal transduction, $A_1$ and $A_2$ adenosine receptors are positively linked to the phospholipase C (PLC) effector system and negatively coupled to the cAMP/protein kinase A effector system, whereas $A_2$ receptors stimulate adenylate cyclase and cAMP formation but may also activate alternative signaling pathways (1.8).

Recently, evidence has been accumulating that $A_1$ and $A_2A$ adenosine receptor agonists in addition to modulating renal blood flow, renin release, and tubular transport (1.5,7) may have potent effects in protecting renal tissue against ischemic reperfusion injury (1.6). In kidneys subjected to ischemia-reperfusion injury, preischemic administration of $A_1$ adenosine receptor agonists induced ischemic preconditioning, whereas posts ischemic $A_2A$ adenosine receptor activation reduced tissue injury by attenuating the reperfusion phase of the injury process (6,9,10). Unlike $A_1$ or $A_2$ receptor agonists, $A_3$ adenosine receptor activation before renal ischemia worsened renal ischemia-reperfusion injury, whereas antagonism of $A_3$ adenosine receptors protected renal function (9).

Looking more closely at the renal actions of adenosine under pathophysiological conditions, adenosine may have several protective effects against renal ischemia-reperfusion injury. Activation of $A_2A$ adenosine receptor agonists, known to attenuate leukocyte accumulation (possibly consequent to decreased endothelial adhesion molecule expression) and to decrease late proinflammatory cytokine expression, may have a potential role in abrogating the inflammatory cascade in ischemia-reperfusion injury (6). Furthermore, treatment of immortalized human proximal tubule cells with $A_1$ or $A_2A$ adenosine receptor agonists correlating with increased cytoprotection against $H_2O_2$ may protect renal tissue against oxygen free radicals, a key mediator of renal perfusion injury (11). Likewise, an increased vasoconstrictor response caused by $A_1$ adenosine receptor activation in the cortex or to sympathetic outflow would reduce renal oxygen consumption, whereas increased renal medullary blood flow caused by $A_2$ adenosine receptor activation would increase it (1.5).
The proximal tubule is the major site of renal Na\(^+\) and HCO\(_3^-\) reabsorption [for review see (12)]. Because of their high basal metabolic demand, proximal tubules located in the outer medulla suffer the most from oxygen deprivation. Downregulation of the renal brush border membrane Na\(^+\)/H\(^+\) exchanger 3 (NHE3), which in the proximal tubule of the kidney mediates absorption of a significant fraction of filtered NaCl and NaHCO\(_3^-\) [reviewed in, e.g., (13,14)] therefore would improve the oxygen supply/demand ratio of proximal tubules under conditions of hypoxia. Despite the effort of recent investigations into the role of A\(_1\) adenosine receptors as drug targets for adenosine receptor antagonists (thought to play a role as diuretics or aimed at ameliorating impairment of tubular Na\(^+\) absorption correlated to progression of renal failure), data supporting a role of A\(_1\) adenosine receptors in protecting the proximal tubule from hypoxic injury via modulation of proximal tubule NaCl or NaHCO\(_3^-\) transport are scant and controversial. In the \textit{in vitro} perfused rabbit proximal convoluted tubule, A\(_1\) adenosine receptor antagonists were found to inhibit basolateral Na\(^+\)-HCO\(_3^-\) symport (15). In opossum kidney (OK) cells, which express a proximal tubule phenotype, stimulation of A\(_1\) adenosine receptors coupled with activation of sodium-dependent glucose and phosphate transport (16). Similarly, studies addressing the physiologic responses to chronic upregulation of A\(_1\) adenosine receptors revealed increased Na\(^+\)-glucose transport in OK cells and in LLC-PK\(_1\) cells, a cell line from pig kidney (17). These observations suggest that endogenous adenosine acting through A\(_1\) adenosine receptors promotes an increase of proximal tubular Na\(^+\) and/or NaHCO\(_3^-\) reabsorption. By contrast to these data, two studies on rat kidney showed that intrarenal application of adenosine decreased tubular Na\(^+\)/H\(^+\) exchange (18,19). Consistent with the hypothesis that inhibition of Na\(^+\) absorption by A\(_1\) adenosine receptors is at least in part effected by inhibition of apical membrane Na\(^+\)/H\(^+\) exchange, we showed in a recent study on A6 cells stably expressing ratNHE3 that activation of A\(_1\) adenosine receptors acutely (within 15 min) inactivates NHE3 and that the effect of A\(_1\) receptors on NHE3 is protein kinase C (PKC) dependent (20).

The observed heterogeneity of adenosine effects on renal Na\(^+\) transport may be due to different experimental conditions in which not-yet-defined cross-talk interactions of signaling cascades mediate A\(_1\) receptor signals. Given the central role of NHE3 in maintaining systemic electrolyte and fluid balance by catalyzing the entry of Na\(^+\) into renal epithelial, we believed that it was necessary to characterize systematically NHE3 regulation during A\(_1\) adenosine receptor activation with the following objectives: (1) to define the mode of regulation of NHE3 activity as a function of time and concentration of A\(_1\) adenosine receptor agonists and (2) to define the relative contribution of the effectors of A\(_1\) receptors (adenylate cyclase [AC], PLC, and Ca\(^{2+}\)) in mediating NHE3 regulation. To accomplish these objectives, we used OK cells, which express an ethylisopropyl-amiloride-resistant apical Na\(^+\)/H\(^+\) exchange activity encoded by NHE3.

**Materials and Methods**

**Materials and Media**

DMEM, PBS, FCS, penicillin and streptomycin, and trypsin-EDTA were purchased from Life Technologies (Karlsruhe, Germany). Cell culture dishes were obtained from Nunc (Karlsruhe, Germany), and flasks were obtained from Sarstedt (Nümbrecht, Germany). Teflon filters Millicell-CM (0.4-µm pore size) were from Millipore (Eschborn, Germany). Collagen R (rat tail) was purchased from Boehringer Ingelheim (Mannheim, Germany). The acetoxymethyl ester of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was obtained from Molecular Probes Europe (Leiden, Netherlands).

Pertussis toxin (PTX), 2-aminoethylidiphenylborate (2-APB), ryanodine, and lanthane (III)-chloride were purchased from Sigma (Munich, Germany) as well as 8-bromo-cAMP, W13, and dopamine. 8-(3-Chlorostyryl)caffeine (CSC), an A2A selective antagonist; 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS 1523), an A\(_3\) antagonist; cytochalasin B; nigerin; and probenecid were also obtained from Sigma. N\(_5\)-cyclopentadenosine (CPA) was purchased from RBI (Taufkirchen, Germany).

8-Cyclopentyl-1,3-dipropylxanthine (CPX), the intracellular calcium chelator BAPTA-AM and U73122, a PLC inhibitor, and its biologic inert analogue U73343 were obtained from Calbiochem (Schwalbach, Germany) as well as the PKC inhibitor calphostin C and the protein kinase A (PKA) inhibitor H89 (N\(_2\)-[p-(Bromocinnamylamino)-o-ethyl]5-isoquinolinesulfonamide). All other chemicals were from Sigma, Merck, or standard commercial sources.

Media used in pH measurements were nominally bicarbonate-free and included isotonic Na\(^+\) medium containing (in mM) 130 NaCl, 4 KCl, 1 CaCl\(_2\), 1 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 18 glucose, and 20 HEPES buffered to pH 7.4 with NaOH; Isotonic TMA\(^+\) medium composed of (in mM) 130 tetramethylammoniumchloride (TMACl), 4 KCl, 1 CaCl\(_2\), 1 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 18 glucose, and 20 HEPES buffered to pH 7.4 with NaOH; Isotonic K\(^+\)-rich medium containing (in mM) 20 NaCl, 110 KCl, 1 CaCl\(_2\), 1 MgSO\(_4\), 1.75 TMAH\(_2\)PO\(_4\), 18 glucose, and 20 HEPES buffered to pH values from 6.5 to 7.72 with NaOH (osmolality, 310 mosmol/kg) and supplemented with 0.5 µM nigericin for calibration of the intracellular BCECF signal.

**Cells and Cell Culture**

Experiments were performed with OK cells, originally obtained from Dr. D. G. Warnock (San Francisco, CA). For experiments, a subclone, selected on the basis of expression of PTH-sensitive Na\(^+\)/phosphate co-transport, was used (21). Cells were cultured in a mixture of DMEM/Ham’s F12, supplemented with 10% FCS, 2 mM glutamine, and 50 lU/ml penicillin and 50 µg/ml streptomycin. Cultures were incubated in a humidified 95% air-5% CO\(_2\) atmosphere at 37°C and subcultured weekly by trypsinization using 0.1% trypsin/0.5 mM EGTA in PBS. Cells generally reached confluence within 3 to 4 d, and experiments were conducted 1 to 2 d after confluence. Studies on OK cells were performed between passages 23 and 57.

**Measurement of Na\(^+\)/H\(^+\) Exchange Activity**

Cellular Na\(^+\)/H\(^+\) exchange activity in OK cells was determined by microfluorimetry using the intracellular pH-sensitive dye BCECF, as described previously (22). Briefly, confluent OK cells grown on permeable support (collagen-coated coverslip with a 1.5-mm hole in the center covered by a Millicell-CM 0.4-µm pore size Teflon filter) were incubated at room temperature for 60 min with 4.2 µM BCECF-AM in Na\(^+\) medium containing 50 µM probenecid to minimize possible dye leakage. Dye-loaded cells were mounted into a perfusion chamber and continuously superfused with Na\(^+\) medium.
(from both the apical and the basolateral compartments) on the stage of an inverted microscope (Zeiss Axiosvert 100) at the rate of 1 ml/min. After recording of baseline intracellular pH (pHi), cells were acid loaded by application of 40 mM NH₄Cl (in Na⁺ medium). Subsequent removal of NH₄Cl and perfusion with sodium-free TMA medium caused the pHi to decrease. Once a stable fluorescence signal was reached, Na⁺ medium was added to the apical cell surface of OK cells, which induced alkalinization of cells. Na⁺/H⁺ exchange activity was calculated from the initial rate of Na⁺-dependent pHi recovery as detailed previously (23).

For assessment of an effect of a test substance on rate of Na⁺/H⁺ exchange activity, pH recovery rates of a single cell layer were recorded under control conditions and after repeated cell acidification under test conditions. Before adopting this protocol for current studies, we verified that rates of pHi recovery after consecutive NH₄Cl pulses were virtually identical when examined from the same starting acid pHi value.

Microfluorometry was performed on a Zeiss Axiosvert 100 inverted microscope equipped with a Zeiss LD “Achroplan” 63 × 0.75 objective, coupled to a multiwavelength illumination system (Polychrom II from T.I.L.L. Photonics, Gräfelfing, Germany). A monochromatizing device with an integral light source (75-W Xenon arc lamp) alternatively selected the excitation wavelengths (495 ± 10 and 440 ± 10 nm) via a galvanometric scanner. The excitation light was directed to the cells via a 515-nm dichroic mirror, and fluorescence emission was collected by a 535 ± 25-nm band-pass filter. Data were recorded either every 6 s or every 30 s by irradiating the cells for 20 ms at each wavelength. The fluorescence signal was captured by a photomultiplier, then downloaded to a computer on which a software program called MetaFluor from Visiiton Systems (Puchheim, Germany) was used to acquire and ratio the two fluorescence signals created by dual excitation. Calibration of the fluorescence signals to pHi cells was performed by using the K⁺/H⁺ ionophore nigericin approach as described previously (23).

Statistical Analyses

Results are represented as mean ± SE. Quantitative differences between control and test conditions were assessed statistically by ANOVA. P < 0.05 was considered statistically significant.

Results

Influence of A₁ Adenosine Receptor Activation on NHE3 Activity

Figure 1 illustrates a dose dependence of the effect of the stable adenosine analogue CPA on NHE3 activity. As shown in Figure 1, exposure of OK cells to CPA for 15 min significantly inhibited NHE3 activity at concentrations between 10⁻⁵ and 10⁻⁷ M (the percentage of inhibition at 10⁻⁵ M, 10⁻⁶ M, and 10⁻⁷ M CPA was -39.0 ± 5.9, -31.2 ± 3.2, and -21.5 ± 5.9, respectively). At 10⁻⁸ M CPA, a small but insignificant increase of NHE3 activity (of 4.4 ± 3.5%) was apparent, which reached significance at 10⁻⁹ M CPA (the percentage of activation at 10⁻⁹ M CPA was 24.8 ± 4.9%). As also shown, positive regulation of NHE3 activity was maintained at concentrations lower than 10⁻⁹ M. Noticeably, treatment with 10⁻¹¹ M CPA still caused an increment of approximately 14% of the transport rate seen under control conditions.

Table 1 summarizes the time dependence of the effect of CPA at a CPA concentration of 10⁻⁶ M, which is close to the natural tubule fluid concentration of adenosine (18). As shown in Table 1, NHE3 activity was effectively inhibited already at 15 min of incubation with 10⁻⁶ M CPA. Maximal inhibition of NHE3 (by 39.7 ± 1.3%) was achieved at 45 min, and inhibition persisted at least during 6 h of incubation with 10⁻⁶ M CPA (data not shown). Table 1 also summarizes data from

Table 1. Time dependence of the effect of CPA on NHE3 activity

<table>
<thead>
<tr>
<th>Exposure Time (Min)</th>
<th>Change of NHE3 Activity (%)</th>
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<tbody>
<tr>
<td>15</td>
<td>-31.2 ± 3.2** (5)</td>
</tr>
<tr>
<td>30</td>
<td>-28.7 ± 1.5* (5)</td>
</tr>
<tr>
<td>45</td>
<td>-39.7 ± 1.3* (5)</td>
</tr>
<tr>
<td>60</td>
<td>-28.5 ± 4.3* (5)</td>
</tr>
<tr>
<td>15</td>
<td>+24.8 ± 4.9** (9)</td>
</tr>
<tr>
<td>30</td>
<td>+42.9 ± 6.0* (6)</td>
</tr>
<tr>
<td>45</td>
<td>+39.4 ± 4.5* (4)</td>
</tr>
<tr>
<td>60</td>
<td>+36.0 ± 6.5* (5)</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of change of NHE3 activity of untreated cells and represent the means ± SE: CPA was applied at a concentration of either 10⁻⁶ M or 10⁻⁹ M. The number of experiments, each consisting of a pair of control and agonist-treated cells, is given in parentheses. ** inhibition of NHE3 activity by CPA: +, activation of NHE3 activity by CPA. Significance from control measurements is indicated by asterisks (* P < 0.05; ** P < 0.01, ANOVA).
corresponding experiments performed at CPA $10^{-9}$ M. As shown, NHE3 activity was continually activated within 60 min of incubation with CPA $10^{-9}$ M. Maximal stimulation of NHE3 activity (of 42.9 ± 6.0%) occurred at 30 min of $10^{-9}$ M CPA treatment.

To confirm that acute changes of NHE3 activity in OK cells in response to CPA were indeed mediated via activation of A$_1$ receptors, we examined transport regulation in the presence of the selective A$_1$ adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX). As shown in Figure 2, 10$^{-7}$ M CPX almost completely abrogated both NHE3 inactivation induced by 10$^{-6}$ M CPA and NHE3 activation induced by $10^{-9}$ M CPA. To rule out any significant effect of an activation A$_{2A}$ or A$_3$ adenosine receptors at micromolar CPA concentrations, we also analyzed effects of CPA (10$^{-6}$ M) on NHE3 activity in the presence of the A$_{2A}$ adenosine receptor antagonist 8-(3-chlorostyryl)caffeine (CSC) and the A$_3$ adenosine receptor antagonist MRS 1523, 3-propyl-6-ethyl-5-[(ethylthio) carbonyl]-2 phenyl-4-propyl-3-pyridine caboxylate, respectively. CSC alone had no significant effect on NHE3 activity (percentage of change CSC $10^{-7}$ M, 20 min, −7.2 ± 6.6 $n = 5$). In the combined presence of CSC and CPA, we observed a small but significant reduction of the inhibitory response of NHE3 to CPA (percentage of change CPA $10^{-6}$ M, 15 min, −31.2 ± 3.2, $n = 5$) with the change induced by CSC $10^{-7}$ M, 20 min and CPA $10^{-6}$ M, 15 min, −23.2 ± 4.0%, $n = 5$, $P < 0.05$). MRS 1523 was inefficient to antagonize the effect of CPA (percentage of change MRS 1523 $10^{-8}$ M, 18 min, 3.8 ± 11.0%, $n = 5$; percentage of change CPA $10^{-6}$ M, 15 min, −31.2 ± 3.2, $n = 5$ compared with the change induced by MRS 1523 $10^{-8}$ M, 18 min plus CPA $10^{-6}$ M, 15 min, −33.5 ± 4.0%, $n = 5$). On the basis of the above results and particularly the findings that only a small percentage of the CPA effect at micromolar concentrations can reliably be attributed to an action at A$_{2A}$ receptors (CSC slightly suppressed NHE3 activity), it is concluded that A$_{2A}$ adenosine receptors have only a small impact on NHE3 control at micromolar CPA concentration.

**Characterization of Signaling Mechanism Accounting for the Inhibitory Action of CPA**

The bimodal effect of CPA suggests the involvement of at least two different signaling cascades. In all studies aiming to identify the mechanism that contributes to NHE3 inactivation, we used a submaximally inhibitory dose of 10$^{-6}$ M CPA, which enables detection of additive effects of agonists, which under conditions of maximal inhibition of NHE3 would escape detection. To compare and validate the results of those studies, we selected to assess responses from cells that were treated for 15 min with CPA. As shown in Figure 3A, a 4-h pretreatment with PTX (200 ng/ml) completely prevented CPA-induced inhibition of NHE3. H89, an inhibitor of PKA, had no effect on downregulation of NHE3 (Figure 3A). U73122, an inhibitor of PLC, almost completely abrogated the inhibitory effect of $10^{-6}$ M CPA, whereas the corresponding biologic inert analogue had no significant effect on CPA-mediated NHE3 inhibition (Figure 3A). Taken together, these results strongly indicate that the inhibitory action of A$_1$ receptors is mediated through activation of PLC and that constitutively active PKA is not required for A$_1$ adenosine receptor–dependent inactivation of NHE3.

The next series of experiments was designed to explore the possibility of whether an elevation of intracellular calcium is required for A$_1$ adenosine receptor–dependent downregulation of NHE3. Figure 3B shows that the inhibitory effect of $10^{-6}$ M CPA on NHE3 activity was completely prevented in cells that were preloaded for 1 h with 2 × 10$^{-5}$ M BAPTA (used to chelate intracellular calcium). By contrast, the typical response of NHE3 to $10^{-6}$ M CPA was barely altered by a 3-min pretreatment with 10$^{-4}$ M LaCl$_3$ (Figure 3B). This suggests that influx of extracellular calcium is not a necessary prerequisite for the change of NHE3 regulation to occur in response to $10^{-6}$ M CPA. Inclusion of 10$^{-4}$ M 2-APB, a permeable modulator of inositol (1,4,5)-trisphosphate–induced Ca$^{2+}$ release, into experiments in the simultaneous presence of $10^{-6}$ M CPA almost completely abrogated inhibition of NHE3 by CPA (Figure 3B). A similar result was observed in experiments that used ryanodin, an endoplasmic ryanodin-sensitive Ca$^{2+}$ store blocker (data not shown). Jointly, these results provide pharmacologic evidence that elevated intracellular Ca$^{2+}$ contributes to inactivation of NHE3 by $10^{-6}$ M CPA.

One target of increased intracellular calcium ([Ca$^{2+}$]$_i$) is calmodulin (CaM), which regulates a variety of kinases and phosphodiesterases as well as basal NHE3 activity (24). Therefore, it was attractive to study whether the CaM antagonist W13 would affect the ability of CPA to control NHE3 activity.

![Figure 2. Antagonism of CPA-induced NHE3 regulation by the A$_1$ adenosine receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (CPX). Data represent the means ± SE of agonist-invoked changes of pH, recovery rates. Exposure to agonists was as follows: CPA $10^{-9}$ M or CPA $10^{-9}$ M, 15 min; CPX $10^{-7}$ M, 20 min. In experiments evaluating the combined effect of CPA and CPX, CPX was added 5 min before the inclusion of CPA into experiments. The number of experiments performed under identical experimental conditions is given in parentheses. **$P < 0.01$ treated versus control; *$P < 0.05$, **$P < 0.01$, treated with CPA plus CPX versus treated with CPA.](image-url)
We found that co-incubation with $5 \times 10^{-6}$ M W13 reduced part of the inhibitory CPA effect (percentage of change CPA $10^{-6}$ M, 15 min, $-31.2 \pm 3.2$ % compared with the change induced by CPA $10^{-6}$ M plus W13 $5 \times 10^{-6}$ M, 15 min, $-18.1 \pm 1.6$; $n = 5$, treated versus control $P < 0.05$; CPA plus W13 versus CPA $P < 0.05$). However, with regard to assigning a definitive role to CaM in the A<sub>1</sub> adenosine receptor pathway, it would be necessary to compare NHE3 regulation in the presence or absence of a higher concentration of the CaM inhibitor. This could not be accomplished, because compared with the nonsignificant effect of $5 \times 10^{-6}$ M W13 (percentage of change of NHE3 activity W13, 15 min, $7.2 \pm 4.7$; $n = 7$), higher concentrations of the CaM inhibitor induced a significant reduction of basal NHE3 activity (data not shown).

The next series of experiments considered the question of whether CPA could lead to an activation of calcium-dependent kinases such as PKC. Induction of PKC activity by CPA or phorbol 12-myristate 13-acetate (TPA) has recently been implicated in CPA-evoked downregulation of transfected NHE3 expressed in the A6 cell line (20). It is interesting that as shown in Figure 3C, co-incubation with the PKC inhibitor calphostin C ($10^{-8}$ M for 15 min) provoked a significant activation of OK-NHE3 activity (by 25.0%; see Figure 3C), whereas co-treatment with TPA ($10^{-7}$ M for 15 min) increased the percentage of inhibition of NHE3 evoked by CPA. Given that the interaction of calphostin C with the regulatory domain of PKC is resistant to the addition of an excess amount of Ca<sup>2+</sup> (25) and considering that TPA is able to suppress local Ca<sup>2+</sup> oscillations, whereas PKC inhibitors are able to prevent this effect (26,27), the data presented in the Figure 3, B and C, are suggestive for a model in which CPA-induced regulation of NHE3 depends on the combined effect of calcium and PKC. In this model, induction of PKC activity is considered necessary for the inactivation of NHE3, whereas a calcium-sensitive but PKC-independent pathway is critical for upregulation of NHE3 activity. On the basis of these considerations, TPA would modulate NHE3 activity through suppression of local elevation of Ca<sup>2+</sup>, whereas calphostin C–dependent suppression of the activity of PKC would allow signal transduction to occur through the stimulatory, calcium-mediated pathway. Additional support of this model is presented in experiments detailed below.

**Characterization of Signaling Mechanism Accounting for the Stimulatory Action of CPA**

As described above, CPA at concentrations of $10^{-9}$ M and lower caused significant activation of NHE3 activity. Because A<sub>1</sub> receptors are negatively coupled to the AC-PKA pathway and NHE3 is sensitive to a reduction of cAMP formation (28), it was conceivable that the observed activation of NHE3 activity could be secondary to inhibition of adenylate cyclase activity. To evaluate this possibility, we compared regulation of NHE3 activity before and after treatment with agents that interfere with the activity of signaling intermediates of the AC-PKA effector system. First, we established that the stimulatory effect of CPA was inhibitable by PTX (average percentage of change of NHE3 activity in response to $10^{-9}$ M...
CPA, 15 min, after a 4-h pretreatment with 200 ng/ml PTX, 3.19 ± 8.79% n = 6). Next, 8-bromo-cAMP was examined for its effect on NHE3 activity. As shown in Figure 4A, pharmacologic activation of PKA by 8-bromo-cAMP (10^{-4} M for 15 min) inhibits NHE3 activity (by 40.0 ± 3.4%). As also shown, co-incubation with 10^{-9} M CPA partially reduced 8-bromo-cAMP-induced inhibition of NHE3 activity. In the presence of 10^{-6} M CPA and 8-bromo-cAMP, the percentage inhibition was lower than that obtained in the presence of 8-bromo-cAMP alone. As illustrated in Figure 4A, activity changes under these conditions resembled that of cells examined for effect of 10^{-6} M CPA alone. An analogue response pattern of NHE3 was seen with dopamine, which inhibits OK-NHE3 activity through a mechanism involving DR1 receptor–mediated PKA activation, whereas stimulation of DR2 receptors has an effect only on NHE3 phosphorylation (29). As depicted in Figure 4B, 10^{-5} M CPA partially abrogated negative regulation elicited by 10^{-5} M dopamine, whereas 10^{-6} M CPA caused only a slight attenuation of the inhibitory effect of dopamine. Together, these observations are interpreted as indicating that A_1 adenosine receptor–dependent control of NHE3 is associated with downregulation of adenylate cyclase activity, most likely triggering a reduction of NHE3 responsiveness to PKA agonists.

The next series of experiments was designed to evaluate the possibility of whether the ability of CPA to stimulate NHE3 activity is due to an activation of effectors of the PLC pathway talking to the AC effector system. The results of these studies are summarized in Figure 5. As shown in Figure 5, incubation with the PLC inhibitor U73122 completely blocked activation of NHE3 seen in the presence of 10^{-9} M CPA alone. Furthermore, preincubation with BAPTA effectively prevented CPA-dependent activation of NHE3, which indicates that elevation of Ca^{2+} is essential for the induction of the stimulatory effect.
of CPA. As also shown in Figure 5, calphostin C was unable to negate positive regulation of NHE3 by the stimulatory dose of CPA, indicating that stimulation of NHE3 does not occur through the effector PKC. To substantiate further that the ability of CPA to attenuate the response of NHE3 to agonists of the AC-PKA system may arise from a cross-talk between elevated calcium and adenylyl cyclase, we examined the combined effect of $10^{-6}$ M CPA and 8-bromo-cAMP in cells that were preexposed to the calcium chelator BAPTA. As reported above and also shown in Figure 5, CPA significantly attenuated the inhibitory response elicited by 8-bromo-cAMP (by 38.2%). Of interest, preincubation with BAPTA blocked the ability of CPA to reiate the inhibitory effect of 8-bromo-cAMP but did not affect the responsiveness of NHE3 to 8-bromo-cAMP. Thus, on the basis of these observations, there is evidence to suggest that stimulation of NHE3 in the presence of the stimulatory dose of CPA is mediated by a mechanism that relies on Ca$^{2+}$-induced inactivation of AC.

Next, we carried out experiments to elucidate the signaling mechanisms that account for CPA-induced stimulation of NHE3 in the presence of the PKC antagonist calphostin C. As shown in Figure 6, CPA-induced upregulation of NHE3 in the presence of calphostin C was completely prevented by inclusion of the A1 adenosine receptor antagonist CPX into perfusion media. Addition of 8-bromo-cAMP effectively abrogated positive regulation of NHE3. Furthermore, preincubation of cells with the Ca$^{2+}$ chelator BAPTA blocked activation of NHE3 in response to co-incubation with CPA and calphostin C. In concert with preceding data, the current findings support a concept in which the A1 adenosine receptor–induced inactivation of NHE3 is mediated by calcium-mediated activation of PKC, whereas stimulation of NHE3 is mediated by a PKC-independent mechanism, in which cytoplasmic calcium is critical for inactivation of adenylyl cyclase. Stimulation of NHE3 as observed in one of our earlier studies on mechanism of the effect of 1,25-dihydroxyvitamin D$_3$ on NHE3 activity (28) may then be the result of a reduction of cellular cAMP formation.

**Effect of Cytochalasin B on CPA-Induced Regulation of NHE3**

Recent evidence suggests that elevated Ca$^{2+}$ inhibits NHE3 activity in PS120 fibroblasts through oligomerization and endocytosis of NHE3, which occurs via formation of an NHE3-E3KARP-α-actinin-4 complex (30). Actinin is an actin-binding protein that cross-links F-actin filaments and also connects F-actin to the plasma membrane. Because modifiers of the actin cytoskeleton, such as cytochalasin B, inhibit NHE3 (31), and actinin may regulate Ca$^{2+}$-dependent endocytosis of NHE3 via linking NHE3 to the cytoskeleton, we wondered how cytochalasin B would influence CPA-dependent regulation of NHE3. Cytochalasin B ($10^{-6}$ M, for 15 min) per se had an inhibitory effect of 22.3 ± 2.8% on NHE3 (six independent observations; $P < 0.05$). Co-incubation with cytochalasin B prevented the suppressive effect of $10^{-6}$ M CPA; its activity after correction for the inhibitory effect of cytochalasin B was −3.7 ± 6.4% of that obtained under control conditions (cells
treated with CPA alone; see Figure 7). Thus, in the presence of cytochalasin B, 10^{-6} M CPA is no longer able to exert an inhibitory action on NHE3. In contrast and also shown in Figure 7, cytochalasin B did not influence CPA-evoked upregulation of NHE3 activity. The percentage of change in the combined presence of 10^{-9} M CPA and cytochalasin B after correction for the effect of cytochalasin B was almost identical to the change of NHE3 activity measured in the presence of 10^{-9} M CPA alone. For control, we studied also the effect of cytochalasin B on phorbol ester–induced inactivation of NHE3 and found that TPA at 10^{-7} M still had an effect on NHE3, although the inhibition in the presence of cytochalasin B was reduced by $-9.5 \pm 3.7\%$. Together, the current data are interpreted as indicating that the cytoskeleton has a role in both, CPA- and TPA-induced downregulation of NHE3 activity, whereas it is not required for CPA-induced upregulation of NHE3 activity.

### Discussion

Previous reports using A6 cells stably expressing a transfected version of rat NHE3 cDNA support a role of adenosine receptors in regulation of NHE3 (20,22). For gaining additional insight into the signaling mechanisms, the current study uses OK cells, a well-established model system for study of the functional regulation of renal brush border membrane NHE3. Our results demonstrated that CPA evoked a bimodal control of endogenous plasmalemmal NHE3 activity: CPA concentrations higher than 10^{-8} M induced inhibition, whereas concentrations lower than 10^{-8} M CPA provoked activation of apical membrane NHE3 activity. The magnitude of responses depended on the concentration of CPA, and both stimulation and inhibition of NHE3 was almost completely prevented by CPX, a specific A1 adenosine receptor antagonist, suggesting involvement of A1 adenosine receptor activation in CPA-dependent control of NHE3 activity. We also demonstrated that the increase and decrease of NHE3 activity induced by CPA was inhibited by PTX. This supported a role of G_{i/0} proteins as intermediates in A1 adenosine receptor–evoked control of NHE3 activity. Furthermore, we presented evidence that up- and downregulation of NHE3 activity elicited by CPA was entirely dependent on PLC activation and release of calcium from intracellular stores: (1) CPA responses of NHE3 were sensitive to the PLC inhibitor U73122; (2) preincubation with BAPTA-AM (chelator of intracellular calcium) prevented CPA-mediated up- and downregulation of NHE3 activity; (3) 2-APB, a modulator of inositol (1,4,5)-trisphosphate–induced Ca^{2+} release, blocked the ability of CPA to modulate NHE3 activity; and (4) LaCl3, an unspecific blocker of Ca^{2+} influx, did not affect regulation of NHE3 elicited by CPA.

Because previous reports indicated involvement of G_{i/0} proteins and PKC in A1 adenosine receptor–mediated attenuation of renal oxidant injury in cultured human proximal cells (11) and presented data documenting that these proteins are intermediate signaling proteins that are also involved in A1 adenosine receptor–mediated protection of rat kidney from ischemic reperfusion injury (32), we elucidated whether PKC activation had an impact on dual control of NHE3 activity in OK cells in response to CPA. We provide evidence that the effector PKC participates in the inhibitory but not the stimulatory control of NHE3 induced by CPA. Our results demonstrate that inhibition of PKC not only blocked negative regulation of NHE3 activity but also allowed activation of NHE3 activity in the presence of the high dose of CPA. In addition and in common with the stimulatory effect of the low dose of CPA, the evoked increase in NHE3 activity in the presence of the PKC inhibitor was dependent on a CPA-induced rise of intracellular Ca^{2+}. Moreover, the ability of CPA to exert a suppressive effect on 8-bromo-cAMP–induced NHE3 inhibition was similarly inhibited by the calcium chelator BAPTA in cells that were incubated with or without the PKC inhibitor. In concert, these results provide strong pharmacologic evidence that activation of PKC is required to mediate the inhibitory CPA effect.

In view of the bimodal effect of CPA and the data that document coupling of the A1 adenosine receptor signal to PLC and elevated Ca^{2+}, what might be the mechanism in the A1 adenosine receptor pathway that transforms the Ca^{2+} signal into stimulatory signal? Although indirect, the evidence that CPA-induced upregulation of NHE3 activity relies on inhibition of adenylyl cyclase activity and that there is a linkage between elevated Ca^{2+} and AC came from the observations that CPA at a low dose attenuated the efficiency of dopamine to inhibit NHE3 activity and, most notably, at a high dose, instead of increasing the degree of NHE3 inhibition, caused a

![Figure 7](Image)
decrease of the inhibition of NHE3 activity evoked by dopamine. Because all of the regulatory effects of CPA were suppressed by preincubation with BAPTA, we speculate that one intriguing role of calcium in the pathway of A1 adenosine receptor–dependent control of NHE3 activity may be that of a sensor of the adenosine concentration. Currently, it is not known which subtype of AC is involved in CPA-induced activation of NHE3. As found by Northern blot analysis, at least five AC isoforms are expressed in rat kidney (type IV, type V, type VI, type VII, and type IX) [for review, see (33)]. Of these isoforms, types V and VI are inhibitable by calcium, and type VI mRNA is expressed along the entire nephron, whereas type V mRNA is restricted to the glomerulus and the initial portion of the collecting duct (34). Although, there is not definite evidence for it, the best hypothesis to explain the attenuating effect of CPA on dopamine-evoked NHE3 inhibition is a CPA-dependent suppression of AC, most probably type VI AC in the case of the low dose of CPA, whereas overall depression of AC activity may explain the effects exerted by micromolar concentrations of CPA. The latter speculation is consistent with results documenting that all forms of AC are sensitive to very high concentrations of intracellular Ca2+ (in the submillimolar range) [for review, see (33)].

Whether Ca2+ exerts positive or negative effects on its effectors directly or via a calcium-binding protein remains to be determined. Among the AC isoforms expressed in the kidney, there is evidence to suggest that type IX AC is inhibited by the activity of the Ca2+/calmodulin-activated protein phosphatase 2B (calcineurin) (35). In the current study, we tested also for possible involvement of calmodulin in the pathway of regulation of NHE3 activity by CPA. We found that W13 reduced part of the inhibitory CPA effect. However, on the basis of our data, we would not like to speculate on the role of CaM in mediating the inhibitory CPA effect, because at the concentration required to completely block the CPA effect, W13 induced a substantial suppression of basal NHE3 activity (data not shown). Currently, other approaches are considered to evaluate the role of CaM and other candidate Ca2+-binding proteins in CPA-dependent control of NHE3 activity. In this context, it is worth mentioning that Levine et al. (24) proposed a role for CaM as a co-factor supporting physiologic NHE3 activity. Contrary to our findings, these authors observed that W13 induced activation of transfected NHE3 in PS120 fibroblasts.

Although the precise mechanism of control of NHE3 activity by A1 receptor activation remains to be defined, one could envisage that shuttling of NHE3 between the plasmalemmal and endosomal membranes may well be responsible for this effect. It was recently shown that endocytosis of NHE3 occurs primarily via clathrin-coated pits (36,37) and that clathrin-mediated endocytosis requires an intact actin cytoskeleton (38). In support of trafficking, we demonstrated that the decrease of NHE3 activity induced by CPA is inhibited by cytochalasin B, which induces cytoskeletal disorganization (31) and reportedly has per se an inhibitory effect on NHE3 (31) (for effect of cytochalasin B, see also the Results section). We further found that whereas the actin cytoskeleton played a role in downregulation of NHE3, cytochalasin B had no effect on CPA-induced upregulation of NHE3 activity. The failure of cytochalasin B to prevent upregulation of NHE3 is not without precedent. For example, in OKP cells, cytochalasin D treatment did not affect endothelin-1–induced NHE3 trafficking, whereas lactrunculin B, which is a more potent inhibitor of the actin cytoskeleton than cytochalasin B or D, inhibited exocytotic insertion of NHE3 (39). These findings were interpreted as indicating that cytochalasin D induced incomplete actin depolymerization, yet in the present study, cytochalasin B attenuated NHE3 inhibition evoked by the high dose of CPA. From this point of view, it is unlikely that the lack of effect of cytochalasin B on CPA-induced activation of NHE3 at the low dose of CPA has to do with an inadequate disruption or relative resistance of actin filaments toward cytochalasin B. Additional work will be required to show whether CPA-dependent control of NHE3 activity is accomplished by altering the distribution of NHE3 between endomembranes and the surface membrane. In support of adenosine’s having an effect on cycling of NHE3, one recent study in the Caco-2 cell line showed that TPA, an agonist of PKC, regulates NHE3 acutely by changing the intrinsic NHE3 activity as well as the number of NHE3 surface protein (40).

The bimodal manner of control of NHE3 activity, with one peak of activity change at micromolar concentrations and the other at nanomolar concentrations, may have some relevance for the validation of the heterogeneity of reported results on A1 adenosine receptor–induced change of function of systems involved in renal Na+ transport. On the basis of our results, it can no longer be assumed that A1 adenosine receptor activation will always increase or decrease transepithelial Na+ transport. About the relative importance of the inhibitory or stimulatory pathway to adenosine regulation of renal apical NHE3 activity, we can only speculate. Under physiologic conditions in which local production of adenosine is low, it is likely that the signal is primarily transduced through inactivation of AC activity leading to activation of NHE3 activity. One recent report showed that adenosine promotes synthesis of renal autacoids, e.g., dopamine (41). From this result and the present findings that CPA antagonizes dopamine-induced inhibition of NHE3, a role of adenosine in Na+ homeostasis may be that of a direct and indirect modulator of renal apical NHE3 activity. The PKC pathway is thought to play a more significant role in modulating NHE3 activity in certain pathophysiologic conditions (e.g., hypoxia, ischemia) in which local production of adenosine is high [for review, see (8)]. By inhibiting NHE3 in particular under these conditions, adenosine could help to improve the oxygen demand and so reinstate the balance of energy supply: demand especially in the S3 segment of the proximal tubule, which is the primary site of injury in renal ischemia (42,43).

In summary, we have shown that A1 adenosine receptor activation contributes to regulation of NHE3 activity and that acute modulation of NHE3 involves the signaling intermediates PLC, Ca2+, PKC, and a yet-to-be-defined effector mediating the stimulatory signal of CPA-induced inhibition of AC on NHE3. We have also shown that, depending on the concentration, A1 adenosine receptor activation by adenosine may induce either up– or downregulation of NHE3 activity. These findings may have potential clinical relevance in preoperative care of patients subjected to renal surgery. Ongoing studies
aimed at identifying C-terminal domains of NHE3 required for CPA-dependent control of NHE3 in combination with studies on change of NHE3 surface protein abundance will shed some additional light on mechanisms whereby A1 adenosine receptor activation controls NHE3 function.

Acknowledgments
This work was supported by the Deutsche Forschungsgemeinschaft, DFG (HE 2416/2-1 to C.H.-K.).

References