Regulation of Na⁺/H⁺ Exchange by Diadenosine Polyphosphates, Angiotensin II, and Vasopressin in Rat Cortical Collecting Duct

Eberhard Schlatter, Sabine Haxelmans, Ieva Ankorina, and Robert Kleta

ABSTRACT

In principal cells of rat cortical collecting ducts (CCD) cellular pH (pHᵢ) is regulated by basolateral Na⁺/H⁺ exchange. The influence of various agonists on pHᵢ and cellular Ca²⁺ activity ([Ca²⁺]ᵢ) in freshly isolated CCD cells was examined with BCECF and fura-2 fluorescence ratios. The recovery of pHᵢ per minute (ΔpH/min) after an acid load was 0.26 ± 0.03 (N = 53) in control conditions and was increased by the diadenosine polyphosphates Ap₄A, Ap₅A, Ap₆A, the phorbol ester phorbol 12-myristat 13-acetate (PMA) (each 5 μmol/L) and angiotensin II (100 nmol/L) by 0.05 ± 0.02 (N = 10), 0.11 ± 0.05 (N = 13), 0.09 ± 0.02 (N = 24), 0.10 ± 0.03 (N = 7), and 0.09 ± 0.03 (N = 8), respectively. Vasopressin (10 nmol/L) decreased ΔpH/min by 0.11 ± 0.03 (N = 9); ATP and Ap₆A (each 5 μmol/L) had no significant effect. The increase in ΔpH/min with Ap₆A was abolished in the presence of an inhibitor of protein kinase C, calphostin C (0.1 μmol/L, N = 8). Fura-2 fluorescence ratio was not significantly changed with angiotensin II, Ap₅A, or Ap₆A but increased with vasopressin, ATP, Ap₅A, and Ap₆A by 0.08 ± 0.02 (N = 13), 0.04 ± 0.02 (N = 13), 0.03 ± 0.01 (N = 14), and 0.03 ± 0.01 (N = 10), respectively. These data indicate that Na⁺/H⁺ exchange in rat CCD is activated by the stimulation of a Ca²⁺-independent protein kinase C and inhibited by protein kinase A.

Key Words: Protein kinase A, protein kinase C, BCECF, fura-2, HOE-694

The intracellular pH (pHᵢ) of principal cells of the cortical collecting duct (CCD) is regulated mostly by the Na⁺/H⁺ exchanger, which extrudes H⁺ in exchange for Na⁺ (1,2). In principal cells of this nephron segment, the Na⁺/H⁺ exchanger is located exclusively in the basolateral membrane (2–4). All of these studies were performed in isolated CCD segments from rabbit kidneys, and no such data from rat CCD are available. Although this transport system seems to be active under basal conditions, it apparently does not contribute significantly to net Na⁺ transport in the rabbit CCD (1) and probably also not in the rat CCD (5). It is, however, generally accepted that pHᵢ and therefore the activity of the Na⁺/H⁺ are used as regulators of cell proliferation and development (6–8). This may be especially important for an epithelium that has a fairly high cellular turnover rate, as do the renal tubular epithelia, especially during tubular damage or inflammation.

Na⁺/H⁺ exchange in many epithelia is regulated by a large number of hormones involving different intracellular transduction pathways (9). Angiotensin II (All) modulates Na⁺/H⁺ exchange in opossum kidney cells (10,11) and in isolated proximal tubules from rabbit kidneys (12). The observed stimulation of exchange activity by All is apparently independent of cytosolic cAMP generation (10,11). In proximal LLC-PK1 and OK cells, agonists increasing cellular cAMP down-regulate Na⁺/H⁺ activity (13,14). In the thick ascending limb again, agonists stimulating cytosolic cAMP production decrease Na⁺/H⁺ activity (15), although there seem to be opposite effects of vasopressin (AVP) on the Na⁺/H⁺ exchangers present in the basolateral (activation) and luminal membrane (inhibition) (16). In Madin-Darby canine kidney cells (17) and in intercalated cells of rabbit CCD (18), mineralocorticoids stimulate Na⁺/H⁺ exchange. In contrast to the proximal tubule, in A6 cells, the Na⁺/H⁺ activity is increased by AVP (19).

In general, Na⁺/H⁺ exchange is regulated in renal as well as in other epithelial and nonepithelial cells through different protein kinases including protein kinase A, protein kinase C, and tyrosine kinase (9,20–23). This diversity of regulation is complicated even further by the presence of various isoforms of the Na⁺/H⁺ exchanger in different cells, but also within one cell type (8,9,24–27).

There is increasing evidence that Na⁺/H⁺ exchange activity is involved in essential hypertension (28–30). Cell proliferation, for example, of mesangial cells is also regulated by vasoactive agonists like All or AVP and nucleotides such as ATP and diadenosine polyphosphates (31–34). In this study, the regulation of Na⁺/H⁺ exchange in the principal cells of rat CCD by these agonist was examined.
METHODS

Cells and Solutions

CCD cells were freshly isolated from kidneys of female Wistar rats (body wt. 100 to 200 g; Charles River Wiga, Sulzfeld, Germany) by the enzymatic method customary in our laboratory and described in detail before (35). Isolated CCD clusters of 10 to 15 cells were fixed with a glass pipette in the heated perfusion chamber mounted on an inverted microscope (Axiovert 10; Zeiss, Jena, Germany) equipped with a 100× oil-immersion lens. The standard bath solution contained (in millimoles per liter): NaCl, 145; K2HPO4, 1.6; KH2PO4, 0.4; calcium gluconate, 1.3; MgCl2, 1; and D-glucose, 5; pH was adjusted to 7.4. All experiments were performed in a running bath (0.5-mL volume) with an exchange rate of 20 times per minute at 37°C. All standard chemicals used were obtained in the highest available purity from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR), fura-2-AM was obtained from Sigma, and pluronic F-127 was purchased from Calbiochem (Bad Soden, Germany). Diadenosine trisphophate (Ap3A), diadenosine tetraphosphate (Ap4A), diadenosine pentaphosphate (Ap5A), and diadenosine hexaphosphate (Ap6A) were purchased from Sigma. HOE-694 was kindly provided by Hoechst AG (Frankfurt, Germany).

Measurements of pH1

CCD cells were loaded with BCECF with the membrane-permeable ester (BCECF-AM, 1 μmol/L, dissolved in 0.1 g/L pluronic F-127 in standard solution) at room temperature for 45 min in the dark. Loaded CCD cells were excited at 488 and 436 nm with a filter wheel (Physiologisches Institut, Universität Freiburg, Germany) rotating at 10 Hz and a xenon-quartz lamp (XBO 75 W; Zeiss) as the light source. Fluorescence at 520 to 560 nm was detected with a single-photon counting tube (H3460-04; Hamamatsu, Herrsching, Germany), and 10 consecutive datum points were averaged, yielding a time resolution of 1 Hz. The autofluorescence and the system noise were measured before the experiment and subtracted from the BCECF fluorescence signals. An iris diaphragm allowed the reduction of the area of measurement to a single cell. Measurements of pH were exclusively taken from those cells demonstrating a low fluorescence typical for principal cells (36). The fluorescence ratio was recorded after a 15-min equilibration period in the continuously perfused bath. Experiments were controlled and data were analyzed with an AT-486 computer system and specific software (U. Fröbe, Universität Freiburg, Germany).

The calibration of the BCECF fluorescence signal was done in 12 separate experiments with the protonophore carbonyl cyanide m-chlorophenyl-hydrazone (CCCP; 1 μmol/L) and external pH values between 6.5 and 8.0 as described in detail before (36). In this range, the dependence of the fluorescence ratio on the external pH was linear and followed the equation pH = 0.86 × ratio + 5.74.

Measurements of Intracellular Ca2+

The intracellular Ca2+ ([Ca2+]i) activity of CCD cells was measured with the Ca2+-sensitive dye fura-2 as described previously (37). CCD cells were incubated with fura-2-acetoxymethyl ester (2 to 5 μmol/L) dissolved with 0.1 g/L pluronic F-127 for 45 min at room temperature in the dark. The incubation was followed by an equilibration period of 15 min while the cells were superfused with control solution at 37°C. Excitation wavelengths were 340, 360, and 380 nm, and the fura-2 emission was recorded at 500 to 530 nm. Measurements were taken from 5 to 10 cells with the aid of an iris diaphragm. The ratio of the emissions after excitations at 340 and 380 nm at 10 Hz was calculated, and 10 consecutive data points were averaged, yielding a time resolution of 1 Hz. Signal noise and autofluorescence were measured and subtracted for each experiment. Fluorescence at 360 nm was used to judge the leakage or bleaching of fura-2, the loss of cells, or air bubbles in the area of measurement during the experiments (37). Calibration of [Ca2+]i was attempted at the end of each experiment by the incubation of the cells with the Ca2+ ionophore ionomycin (1 μmol/L; Sigma, Deisenhofen, Germany) in the presence (1.3 mmol/L) and nominal absence of Ca2+ (with 5 mmol/L ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid present) according to standard methods (38). For those experiments where a paired calibration at the end of the experiment was not successful, mean values of all calibrations were used to estimate [Ca2+]i.

Statistical Analysis

Data are presented as fluorescence ratios (488/436 nm and 340/380 nm for pH1 and [Ca2+]i, respectively) in the figures presenting original recordings or as mean values of calculated pH1 values or fura-2 fluorescence ratios or calculated [Ca2+]i values ± SE (N), where N refers to the number of experiments. A paired or nonpaired t test (two-sided) was used where appropriate to test for statistically significant differences. P = 0.05 was set as the significance level.

RESULTS

Basal Activity of Na+/H+ Exchange

A total of 45 rats were used for this study, and 65 isolated CCD cell clusters were examined. The BCECF fluorescence ratio as an indicator of pH1 was 2.78 ± 0.04 in resting conditions at the beginning of the experiments. By the use of the calibration curve obtained in separate experiments (see Methods), this fluorescence ratio value corresponded to an estimated pH1 of 7.23 ± 0.04 for these principal cells of rat CCD. The inhibitor of the Na+/H+ exchanger HOE-694 (39), specific for the isomeric NHE-1 present in the basolateral membranes of the CCD, was used to estimate the contribution of this exchanger to the basal pH1 of the CCD. HOE-694 acidified CCD cells by 0.02 ± 0.01 (N = 9) and by 0.09 ± 0.02 (N = 7) pH units at concentrations of 1 and 10 μmol/L, respectively.

Recovery From an Acid Load

The addition of 20 mmol/L NH4+ /NH3 to the control solution (pH = 7.4) resulted in a transient increase in pH1 by 0.83 ± 0.05 (N = 64) pH units; the removal of NH4+/NH3 transiently decreased pH1 by 0.62 ± 0.03 pH units (Figure 1). The recovery from such an acidification was impaired in the presence of 1 μmol/L HOE-694. Figure 1 depicts the original recording of such an experiment. The activity of the Na+/H+ exchanger in these cells after NH4+/NH3-induced acidification was estimated as the initial slope (first 120 s) of the increase.
in pH$_1$ after it had reached a minimal value. This recovery rate ($\Delta$pH$_1$/min) was 0.26 ± 0.03 ($N = 53$) under control conditions. In the presence of 1 $\mu$mol/L HOE-694, $\Delta$pH$_1$/min was reduced by 0.13 ± 0.03 ($N = 8$).

**Absence of Effects of Agonists on Basal pH$_1$**

The influence of various agonists on basal pH$_1$ was examined by exposing CCD cells to them for 3 to 5 min in the constantly perfused chamber. None of the tested agonists—All (100 nmol/L; $N = 5$), AVP (10 nmol/L; $N = 6$), ATP (10 nmol/L; $N = 5$), Ap$_3$A (10 nmol/L; $N = 6$), ATP$_6$A (10 nmol/L; $N = 6$), and Ap$_6$A (10 nmol/L; $N = 6$), each 5 $\mu$mol/L—significantly changed pH$_1$ under resting conditions. The stimulation of protein kinase C with the phorbol ester PMA (5 $\mu$mol/L) also did not significantly alter pH$_1$ ($N = 7$), whereas the inhibitor of protein kinase C, calphostin C (0.1 $\mu$mol/L), reduced pH$_1$ significantly by 0.08 ± 0.02 pH units ($N = 7$).

**Influence of Agonists on Recovery From an Acid Load**

The influence of the above agonists on the recovery of pH$_1$ after an NH$_4^+$/NH$_3$-induced acidification was examined as an indication for their ability to change the activity of the Na$^+$/H$^+$ exchanger after it was activated by cytosolic acidification. As demonstrated by the original recordings of the BCECF fluorescence ratio depicted in Figure 2, AVP (10 nmol/L) decreased and All (100 nmol/L) enhanced the recovery of pH$_1$ after transient acidification by NH$_4^+$/NH$_3$. These modulations of Na$^+$/H$^+$ exchange activity by AVP and All amounted on average to an inhibition by 32 ± 11% (AVP; $N = 8$) and an activation by 37 ± 7% (All; $N = 9$) of the respective control values (Figure 3). ATP and the diadenosine polyphosphate Ap$_3$A had no significant effect on pH$_1$ recovery, whereas the dinucleotides Ap$_4$A, Ap$_5$A, and Ap$_6$A significantly accelerated this recovery of pH$_1$. When the inhibitor of protein kinase C, calphostin C (0.1 $\mu$mol/L), was present, the acceleration of pH$_1$ recovery by Ap$_6$A was not only inhibited, but pH$_1$ recovery was even reduced compared with the respective control situation (Figures 4 and 5). The addition of the phorbol ester PMA (5 $\mu$mol/L) led to a similar increase in pH$_1$ recovery, as Ap$_6$A and Ap$_6$A did (Figure 5).
Influence of Agonists on Cellular Ca^{2+} Activity

To examine whether the cellular Ca^{2+} activity is involved in the activation or inhibition of Na^+/H^+ exchange activity by the tested agonists, we examined the influence of these substances on cellular Ca^{2+} activity under resting conditions at normal cellular pH. Whereas AVP (10 nmol/L) increased fura-2 fluorescence ratio transiently, there was no significant effect of All (0.1 μmol/L). The diadenosine polyphosphate Ap₅A (5 μmol/L) increased the fluorescence ratio transiently. These effects are demonstrated in the original recordings depicted in Figure 5. The changes in the fura-2 fluorescence ratio and corresponding calculated changes in [Ca^{2+}]_i for these agonists, and in addition for ATP and the other diadenosine polyphosphates Ap₃A, Ap₄A, and Ap₆A, are summarized in Figure 7.

DISCUSSION

The experiments described demonstrate that in principal cells of rat CCD, Na^+/H^+ exchange activity is low at normal resting pH. After the activation of the exchanger by cellular acidification, a regulation of this transport system by various agonists can be observed.
Regulation of Na⁺/H⁺ Exchange

When cellular pH, was around 7.2, as in resting cells, no significant effects on pH, were detectable when various agonists known to modify Na⁺/H⁺ exchange activity in other preparations were added. This was the case for the protein kinase A activating agonist AVP and for the protein kinase C activating phorbol ester PMA. Again without significant effect on pH, were nucleotides such as ATP and the diadenosine polyphosphates, of which at least Ap₄A, Ap₅A, and Ap₆A probably also activated protein kinase (see below). In addition, AII, which decreases cellular cAMP concentration, at least in proximal OK cells (10), was without effect on basal pH,. An influence of cAMP-activating agonists like AVP, parathyroid hormone, or prostaglandin E₂ (15) and of AII (12) on basal pH, was seen in rabbit proximal and rat medullary thick ascending limb cells, respectively. The absence of effects of the tested agonists on basal pH, in the CCD cells supports the notion that in this situation Na⁺/H⁺ exchange activity is low and that under these conditions the above-mentioned agonists do not significantly alter its activity.

The Na⁺/H⁺ exchanger can be activated by cytosolic acidification, e.g., by using the NH₄⁺ prepulse technique. Under this condition, its activity can be estimated from the slope of the pH, recovery after maximal acidification. Because cells were examined in the absence of HCO₃⁻ and CO₂ in this study, the Na⁺/H⁺ exchanger is the dominating if not the only system available to alkalinate the cells after such an acidification. This pH, recovery in rat CCD cells could be modulated by several agonists. AII, as in other systems (10–12), stimulated Na⁺/H⁺ exchange. AII, again comparable to other cells (9.14–16,19), decreased the activity of the Na⁺/H⁺ exchanger. The latter effect is obviously restricted to the NHE-1 isoform of the antiporter, whereas the NHE-2 isoform is activated by AVP (13). In the principal cell of rabbit CCD, the Na⁺/H⁺ exchanger is restricted to the basolateral membrane (3). Generally, the Na⁺/H⁺ exchanger located in the basolateral membranes of epithelia is the NHE-1 isoform. The activation of protein kinase C by PMA led to an increase in the pH, recovery in these cells, similar to such effects on Na⁺/H⁺ exchange in basolateral membranes of LLC-PK1 cells, MCT cells, and RKPC-2 cells (9). Again, this stimulatory effect of phorbol esters on Na⁺/H⁺ exchange seems to be restricted to the NHE-1 isoform, whereas phorbol esters inhibit the NHE-2 isoform (13). The diadenosine polyphosphates Ap₄A, Ap₅A, and Ap₆A also led to an activation of Na⁺/H⁺ exchange. The fact that in the presence of the inhibitor of protein kinase C, calphostin C, the stimulatory effect of Ap₆A on Na⁺/H⁺ exchange was blunted indicates that these substances exert their action on Na⁺/H⁺ exchange via an activation of protein kinase C. These effects of the diadenosine polyphosphates are most likely mediated via specific receptors and not via common purinergic receptors because no effect of ATP itself was observed. Recently, evidence was provided for the presence of a P₂₄ receptor in rabbit CCD (41). A specific diadenosine tetraphosphate receptor has been described in the heart (42).

Effects of AVP, AII, ATP, and Diadenosine Polyphosphates on [Ca²⁺]

To examine whether the modulation of Na⁺/H⁺ exchange in rat CCD cells by AVP, AII, ATP, and diadenosine polyphosphates was dependent on parallel changes in cellular [Ca²⁺], we examined the [Ca²⁺] after the addition of these agonists. So far, increases in cellular Ca²⁺ activity by hormones in the CCD have only been reported for the rabbit CCD, e.g., for AVP (43,44) and ATP (41), for the rat outer and inner medullary collecting duct for ATP (45), and in the rat terminal inner medullary collecting duct for ATP (46). Here, we demonstrate that AVP and ATP both led to an increase in [Ca²⁺] also in the rat CCD. Whereas AII did not significantly change [Ca²⁺], the diadenosine polyphosphates moderately increased [Ca²⁺], as well. Such an increase in [Ca²⁺], by diadenosine polyphosphates has been demonstrated in a number of cells before, including mesangial cells (47). From these findings, it is apparent that no correlation exists between the effects of these agonists on [Ca²⁺] and Na⁺/H⁺ exchange activity. Because the activation of protein kinase C by the phorbol ester PMA was able to stimulate Na⁺/H⁺ exchange, this activation may involve a Ca²⁺-independent protein kinase C isoform. Recently, the presence of the isoforms α, δ, ε, η, and ι were detected in rat CCD on the mRNA level (48). From these five isoforms, the δ and ε isoforms are Ca²⁺ independent and can be activated by PMA (49). The isoform ε is apparently also involved in the AII-induced activation of protein kinase C in rat mesangial cells (50).

Potential Importance of Agonist-Mediated Na⁺/H⁺ Regulation in the CCD

As mentioned above, Na⁺/H⁺ exchange does not influence transepithelial salt transport in this nephron segment; it is, however, important for the regulation of pH, in these cells, which can be exposed to an acidic luminal pH, especially during metabolic acidosis. Therefore, changes in the acid/base excretion to maintain the acid/base balance of the organism may involve hormonal modulation as well. Potentially more important than such a hormonal regulation of Na⁺/H⁺ in the CCD might be its involvement in cell proliferation. It has been demonstrated that vasoactive substances such as AII are also very potent stimulators of cell proliferation, e.g., in the glomerular mesangial cell (52). The vasoactive diadenosine polyphosphates have recently also been described to stimulate cell proliferation in these cells (34). Cell proliferation always involves an alkalization of cell pH, and an activation of Na⁺/H⁺ exchange during cell proliferation has been demonstrated in a
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varied cells (8,25,40). In renal tubules, an increased cell proliferation is especially important during tubular inflammation or cellular damage. Because substances like the diadenosine polyphosphates are present in high concentrations in platelets (51), they could mediate cell proliferation when they are released from these cells in such a situation.

In conclusion, this study provides evidence that in the principal cells of the rat CTD, Na+/H+ exchange is inhibited by AVP and activated by All and by the stimulation of protein kinase C. In addition, we demonstrated for the first time that diadenosine polyphosphates are capable of activating Na+/H+ exchange. Thus, another important function can be added to the known vasoactive and proliferative action of the diadenosine polyphosphates.

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