Expression of isoforms of the Na\(^+/\)H\(^+\) exchanger in M-1 mouse cortical collecting duct cells

C. HILL, A. N. GIESBERTS, AND S. J. WHITE
Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom

Received 20 September 2000; accepted in final form 31 October 2001

Hill, C., A. N. Giesberts, and S. J. White. Expression of isoforms of the Na\(^+/\)H\(^+\) exchanger in M-1 mouse cortical collecting duct cells. Am J Physiol Renal Physiol 282: F649–F654, 2002; 10.1152/ajprenal.00291.2000.—Na\(^+/\)H\(^+\) exchanger (NHE) proteins perform a variety of functions in the kidney and are differentially distributed among nephron segments. The purpose of this study was to identify NHE isoforms in murine M-1 cells as a model of cortical collecting duct principal cells. It was found that mRNAs corresponding to NHE1, NHE2, and NHE4 are expressed in M-1 cells. NHE-dependent regulation of intracellular pH (pHi) was investigated in the absence of extracellular HCO\(_3^-\). Application of a 20 mM NH\(_4\)Cl pulse resulted in a reversible intracellular acidification from which recovery was partially inhibited by application of 1 mM amiloride to either the apical or the basolateral membranes and was abolished when amiloride was applied to both sides of the monolayers, which suggests that NHEs are expressed in both the apical and the basolateral cell membranes of M-1 cells. The purinergic agonists ATP and benzoylbenzoyl-ATP caused a reduction of pHi when applied to the apical membrane, which suggests pHi may be influenced by extracellular nucleotides in the luminal fluid of the cortical collecting duct.

The renal cortical collecting duct (CCD) in mammals plays an important role in regulating Na\(^+\) absorption and H\(^+\) secretion, thus regulating the NaCl content and pH of urine.

Molecular cloning studies have identified several forms of the mammalian Na\(^+/\)H\(^+\) exchangers (NHEs) 1–5 (29). NHE1–4 have been identified in the kidney. There is evidence for NHE activity in the proximal tubule (12), loop of Henle (9), distal tubule (27), and the cortical and medullary segments of the collecting duct system (5, 10, 28).

NHE1 is expressed ubiquitously among mammalian cells and is localized in the nephron to the basolateral membrane of epithelial cells (2). NHE1 is sensitive to inhibition by amiloride, a nonspecific inhibitor of Na\(^+/\)H\(^+\) exchange, and is involved in controlling intracellular pH (pHi) and in regulating cell volume. NHE2 is also amiloride sensitive, and its cell membrane location may be tissue specific. In intestinal epithelial cells, NHE2 is expressed in brush-border membranes (11). In a cell line from mouse inner medullary collecting duct, NHE2 was localized in the basolateral membrane (22); however, in the pig kidney proximal tubule cell line LLC-PK1, NHE2 was reported to be present in the brush-border membranes (14). NHE3 is resistant to amiloride inhibition and is expressed in brush-border membranes of rat kidney proximal tubule (1) and intestine (11). Expression of NHE4, an amiloride-insensitive isoform, has not been fully explored, but previous work has shown NHE4 to be localized in the basolateral domain of ascending limbs of Henle’s loop, distal tubules, and early proximal tubules (6).

The murine kidney cell line M-1 was derived from dissected CCDs of a mouse transgenic for the early region of the SV40 virus (23). This cell line displays general transport and electrophysiological characteristics of CCD principal cells (13, 16, 17, 23). The mouse is the present animal of choice for the generation of transgenic or targeted gene knockout models. However, compared with other species such as rat or rabbit for many physiological systems, comparatively little functional data exist. To date, little is known regarding either the function or the identity of H\(^+\) and HCO\(_3^-\) transporters in mouse CCD principal cells. In this study, a combination of molecular, biological, and functional approaches was used to determine the presence of non-bicarbonate-dependent mechanisms of regulation of pHi in polarized monolayers of M-1 cells. The results are consistent with the idea that NHE1, NHE2, and NHE4 are expressed in mouse CCD and that, after an intracellular acid load, H\(^+\) extrusion occurs via NHEs at both the apical and the basolateral membranes. The presence of extracellular nucleotides also influenced pHi. The significance of these findings is discussed in relation to the control of Na\(^+\) transport in the CCD.

METHODS

Cell culture. The M-1 cells in this study were a gift from Dr. C. Korbmacher (University Laboratory of Physiology,
Oxford, UK). Cells were used between passages 27 and 35, cultured on either plastic or permeable filter supports (Snapwell; Corning CoStar, High Wycombe, Bucks, UK) and grown to confluence at 37°C in a humidified 95% air-5% CO₂ atmosphere. The cultures were maintained in serum-free media (PC-1; Biowhittaker, Wokingham, UK) supplemented with 2 mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (Sigma, Poole, UK). Media was changed every 2 days, and cells were passaged every 3 days by trypsinization.

Reverse transcription-PCR determination of NHE mRNA expression. All reagents were obtained from Promega (Southampton, UK) unless otherwise stated. Total RNA was extracted from M-1 cells and mouse kidney by using TriZol reagent (GIBCO BRL, Paisley, UK) according to the manufacturer’s instructions and then treated with DNase I to remove genomic DNA. Reverse transcription reactions used 2 μg of total RNA, 2 μl of 25 μM JWI primer [an oligo(dT)(11)], and molecular biology-grade water up to a final volume of 12.9 μl. Samples were heated at 90°C for 2 min to denature RNA secondary structures. RT buffer (4 μl), deoxynucleotide phosphate mix (1.6 μl), and Moloney murine leukemia virus-RT (1.5 μl) were added to each sample and allowed to anneal on ice. Finally, samples were heated for 1 h at 37°C and then for 5 min at 95°C to denature the RT. PCR was performed with primers on the basis of published sequences for the four isoforms (1–5) located near the 3’-end of the coding region, where there is low-sequence homology among the rat NHE isoforms (Table 1) (4). The PCR reaction contained 50 mM KCl, 10 mM Tris·HCl (pH 9.0 at 25°C), 0.1% Triton-X 100, 200 mM deoxynucleotide phosphate mix, 3.5 mM MgCl₂, 1.25 U Taq polymerase, 200 nM of each primer (NHE1, NHE2, NHE3, NHE4, or the “housekeeping gene” glyceraldehyde-3-phosphate dehydrogenase), plus the target DNA.

Table 1. Primer sets for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Predicted Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>TCTGCCGCTGCTGAACTGTC</td>
<td>422</td>
</tr>
<tr>
<td>Sense</td>
<td>CGCTTCAAGCTCTGATTCAGCA</td>
<td></td>
</tr>
<tr>
<td>NHE2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>CGAGATCGTAAATGAGCAGGA</td>
<td>310</td>
</tr>
<tr>
<td>Sense</td>
<td>CTTGGTGGGGGGTGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>NHE3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GGAAACAGGGGGGAGGGGAGCCAT</td>
<td>321</td>
</tr>
<tr>
<td>Sense</td>
<td>GAATCTGTGTTGCGAGATTC</td>
<td></td>
</tr>
<tr>
<td>NHE4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GCTGGGAGGTTGGAGATCCTG</td>
<td>501</td>
</tr>
<tr>
<td>Sense</td>
<td>CTTGGGCCGCTGACAGGTTG</td>
<td></td>
</tr>
</tbody>
</table>

Measurement of pHᵢ. pHᵢ was determined by using the pH-sensitive fluorescent dye [2’,7’-bis-(2-carboxyethyl)-5(6’)-carboxyfluorescein] acetoxyethyl ester (Molecular Probes, Leiden, The Netherlands). Cells were loaded in the presence of 10 μM 2’,7’-bis-(2-carboxyethyl)-5(6’)-carboxyfluorescein, acetoxyethyl ester in HEPES buffer for 30 min at room temperature. Bath superfusion was then commenced, and the bath solution temperature was raised to 37 ± 0.5°C and maintained thereafter. After 30 min, cells were illuminated with alternate excitation wavelengths of 440 and 490 nm and data acquisition commenced. The level of fluorescence was typically 50–200 times the background level. Acquisition and display of the data were controlled by the Felix 1.2 PC software (Photon Technologies).

Calibration of the 440/490 emission ratio was performed by the nigericin method described by Thomas et al. (25). Briefly, at the end of each experiment, the bath was perfused with a high-K⁺ solution at pH 7.4 (in mM) 100 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES]. Nigericin was added at a final concentration of 7 × 10⁻³ mg/ml. The bath was then superfused with solutions of known pH, and the fluorescence ratio was recorded, allowing the fluorescence ratio to reach a steady state at each pH. All reagents, unless otherwise stated, were purchased from Sigma.

Statistics. Values are reported as means ± SE, and n is the number of experiments performed. To compare means, Student’s unpaired or paired t-tests were used as appropriate. For all comparisons, a value of P < 0.05 was considered to be significant. Figures of gels are representative examples.

RESULTS

NHE mRNA expression in mouse kidney and M-1 cells. mRNA was amplified by PCR by using primers designed previously (4) to recognize the COOH terminus of NHE isoforms. This region has low-sequence homology between isoforms but is highly conserved between species (4). As expected, analysis of the PCR products from mouse kidney revealed cDNA products of appropriate sizes (Fig. 1A): 422 bp for NHE1, 310 bp for NHE2, 321 bp for NHE3, and 501 bp for NHE4. In M-1 cells, products of 422 bp for NHE1, 310 bp for NHE2, and 501 bp for NHE4 were observed. However, no product was observed for NHE3 (Fig. 1B). Each reaction was performed with and without RT. In the absence of reverse transcription, no products were observed (Fig. 1, A and B), indicating that genomic DNA did not contaminate the samples. The efficiency of the RNA extraction and RT reactions was confirmed for all samples by amplification of a 597-bp product for glyceraldehyde-3-phosphate dehydrogenase (Fig. 1, A and B).

Functional localization of Na⁺/H⁺ exchange in M-1 cells. The mean pHᵢ in M-1 cells was 7.20 ± 0.11 (n = 23) when both surfaces were superfused with control solution at pH 7.4. After a 20 mM NH₄Cl pulse, the mean reduction in pHᵢ (ΔpHᵢ) was 0.27 ± 0.08 pH units (P < 0.05, n = 17). The mean rate of recovery after acidification was 1.3 ± 0.28 × 10⁻³ pH/s (Fig. 2; n = 8). Application of amiloride, a nonselective inhibitor of
Na⁺/H⁺ exchange, to the apical side reduced the rate of recovery from the acid load to $2.65 \pm 0.37 \times 10^{-4}$ pH/s (Fig. 3A; $P < 0.001, n = 4$). The rate of recovery increased toward control values on removal of amiloride. When amiloride was applied to the basolateral side alone, the rate of recovery from the acid load was reduced to $1.49 \pm 0.8 \times 10^{-4}$ pH/s (Fig. 3B; $P < 0.001, n = 5$). Addition of amiloride to both sides of the monolayers completely abolished pH recovery from an acid load, and pHᵢ continued to fall at a rate of $5.6 \pm 1.9 \times 10^{-5}$ pH/s (Fig. 3C; $P < 0.001, n = 5$) until amiloride was removed, whereupon there was a recovery to control levels. Substitution of extracellular Na⁺ with the membrane-impermeant cation N-methyl-d-glucamine after the acid load also resulted in continued intracellular acidification ($3.2 \pm 1.6 \times 10^{-5}$ pH/s, $P < 0.001, n = 5$), which confirms the dependency of the recovery process on Na⁺/H⁺ exchange.
Extracellular nucleotides produce intracellular acidification in M-1 cells. Transport of both Na\(^+\) and Cl\(^-\) is altered in mouse M-1 cells by extracellular nucleotides (8). Therefore, we tested whether activation of purinergic receptors might lead to alterations in pHi. Figure 4 shows the effects of two P2 agonists on pHi in M-1 cells. Addition of 1 mM ATP (a mixed P2X and P2Y agonist) to the apical superfusate caused a reversible intracellular acidification (Fig. 4A; \(\Delta \text{pHi} = 0.24 \pm 0.09, n = 8, P < 0.05\)). Application of 0.1 mM of 2' and 3'-O-(4-benzoxybenzoyl)-ATP (Bz-ATP), a selective P2X\(_7\) (P2Z) agonist, to the apical superfusate also resulted in a small but statistically significant acidification (Fig. 4B; \(\Delta \text{pHi} = 0.058 \pm 0.02, n = 8, P < 0.05\)). A reduction of pHi was observed only on activation of receptors in the apical membrane. In contrast, application of either ATP or Bz-ATP to the basolateral superfusate was without effect on pHi (data not shown). The response was not mediated by means of the P2Y\(_2\) receptor subtype, because the P2Y\(_2\) agonist UTP was also without effect on pHi, irrespective of the side of application (data not shown).

DISCUSSION

The overall purpose of this study was to determine the functional location of Na\(^+\)/H\(^+\) exchange and to identify particular NHE isoforms that are expressed in M-1 cells as a model of murine CCD principal cells. Compared with the more extensively characterized experimental models of rabbit and rat CCD, there is little information available regarding the functional properties of this nephron segment of mouse kidney. The M-1 cell line displays general transport properties characteristic of principal cells of the CCD (23). M-1 cells express subunits of the amiloride-sensitive Na\(^+\) channel (16), and monolayers display moderate sensitivity to both glucocorticoids and mineralocorticoids (17).

By using RT-PCR, we have determined that M-1 cells express mRNAs for NHE1, NHE2, and NHE4. These results are supported by studies of the rabbit CCD cell line RC.SV3, which expresses an identical NHE profile (10). It is clear that there are highly specific differences in NHE expression between segments of the nephron. Cortical and medullary thick ascending limbs (TALs) of rat kidney have been reported to express both NHE1 and NHE3 but not NHE2 or NHE4 (4). Cells of the macula densa of rabbit kidney express both NHE2 and NHE4 in the apical and basolateral membranes, respectively (19). This expression pattern is similar to that reported for mouse inner medullary collecting ducts (24). There is considerable controversy regarding NHE2 expression in the kidney. One report (3) failed to detect either NHE2 mRNA or protein in the kidney, whereas others (7) have reported that NHE2 is expressed in the apical membranes of cortical and medullary TALs but not in the collecting duct of rat kidney. The present results are consistent with the absence of NHE3 from mouse CCD, because no mRNA was demonstrated by RT-PCR, although the primers used in the study were able to detect NHE3 mRNA from whole mouse kidney. NHE3 is expressed in the apical membrane of the proximal convoluted tubule, where it appears to mediate a significant fraction of Na\(^+\) and water reabsorption (26). In the TAL, NHE3 is expressed in the apical membranes of both cortical and medullary segments; however, this isoform does not appear to contribute to salt reabsorption in the TAL under normal conditions (26), but its activity is increased by chronic metabolic acidosis (15). A question arising from the present study is whether the profile of NHE mRNA expression that we have observed reflects functional protein expression. This is not always known to be the case. As reported previously (20), NHE4 protein is undetectable in brain tissue despite detectable levels of mRNA expression. Such discrepancies may be the result of various mechanisms of posttranscriptional regulation of gene expression. Successful identification of specific NHE proteins in M-1 cells has so far evaded us. We have made exhaustive attempts (Hill C and White SJ, unpublished observations), using a variety of available antibodies raised against NHE isoforms, to determine expression and localization of these proteins by immunoblotting and immunocytochemistry. However, to date, these studies have proven inconclusive because of the lack of specificity of such antibodies.
It is clear that NHE activity is present in both the apical and the basolateral membranes of M-1 cells. Amiloride applied to either the apical or the basolateral membranes of M-1 cells reduced the rate at which pH$_i$ recovered from an intracellular acid load. Although amiloride at the concentration used in this study would also inhibit Na$^+$ entry via the amiloride-sensitive Na$^+$ channel, this would tend to increase the driving force for apical proton extrusion. Simultaneous inhibition of Na$^+/H^+$ exchange at both the apical and the basolateral membranes by amiloride or by removal of extracellular Na$^+$ abolished recovery of pH$_i$ from an intracellular acidification. From these results, it is concluded that, under nominally HCO$_3^-$-free conditions, NHE is the principal mechanism by which pH$_i$ is regulated in M-1 cells. Similarly, in principal cells of rabbit CCD, Na$^+/H^+$ exchange is a major regulator of pH$_i$ (5, 21, 28). In principal cells of the rabbit CCD, the NHE does not contribute to the feedback control of Na$^+$ transport, because alteration of intracellular Na$^+$ concentration does not alter pH$_i$ (21). Whether this is the case in murine CCD principal cells remains to be established. Although our present findings do not allow us to identify with certainty the specific NHE isoforms expressed in the apical and basolateral membranes, our experiments do yield information on NHE4. This NHE isoform is highly resistant to amiloride (6). However, recovery of pH$_i$ from an intracellular acid load was completely inhibited by amiloride. This suggests that, if NHE4 protein is present in M-1 cells, it is not expressed in a functionally active form in plasma membranes.

An observation of additional interest arising from the present study is that ATP reduced pH$_i$ when applied to the apical, but not the basolateral, membrane. ATP (a mixed P2X and P2Y agonist) and the specific P2Z agonist Bz-ATP both produced an intracellular acidification. The decrease in intracellular H$^+$ activity could not have been caused by activation of P2Y$_2$ receptors, because the P2Y$_2$ agonist UTP was without effect. A recent study by Cuffe et al. (8) has shown that activation of apical and basolateral P2Y$_2$ receptors by ATP stimulates Cl$^-$ secretion in M-1 cells while inhibiting amiloride-sensitive Na$^+$ transport. The mechanism of inhibition of Na$^+$ transport by ATP is unknown. Although we have not measured Na$^+$ transport in this study, our results are consistent with the idea that inhibition of Na$^+$ transport by luminal ATP (8) may be in part mediated by means of intracellular acidification. The increase in H$^+$ activity resulting from activation of apical purinoceptors would reduce Na$^+$ entry across the apical membrane by inhibiting activity of the amiloride-sensitive Na$^+$ channel, which is highly pH sensitive (18). Further experiments will be required to directly test this hypothesis. We also found that, although Bz-ATP produced an acidification, it was not as effective as ATP, suggesting that the reduction in pH$_i$ did not result from activation of the P2X$_7$ receptor subtype. None of the nucleotides tested had an effect on pH$_i$ when applied in the basolateral superfuse, even though M-1 cells express functional P2Y$_2$ receptors in the basolateral membrane (8). The polarity of the effect observed here strengthens the suggestion that apical and basolateral purinoceptors are able to act independently of one another in Na$^+$-transporting epithelia (8).

In summary, the results from this study show that the M-1 cell line expresses NHE1, NHE2, and NHE4 mRNAs and that functional Na$^+/H^+$ exchange activity is present at both the apical and the basolateral plasma membranes. Activation of apical purinoceptors leads to a decrease in pH$_i$, which may contribute to the mechanism by which extracellular nucleotides influence Na$^+$ entry across the apical membrane in principal cells of the collecting duct.

We thank Dr. Christoph Korbmacher (University Laboratory of Physiology, Oxford, UK) for the M-1 cells and Andrew J. Parker for general technical support.

This work was supported by the National Kidney Research Fund of the United Kingdom.

REFERENCES


