

## Adenosine inhibits the transfected $\text{Na}^+ - \text{H}^+$ exchanger NHE3 in *Xenopus laevis* renal epithelial cells (A6/C1)

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1. Adenosine influences the vectorial transport of  $\text{Na}^+$  and  $\text{HCO}_3^-$  across kidney epithelial cells. However, its action on effector proteins, such as the  $\text{Na}^+ - \text{H}^+$  exchanger NHE3, an epithelial brush border isoform of the  $\text{Na}^+ - \text{H}^+$  exchanger (NHE) gene family, is not yet defined.
2. The present study was conducted in *Xenopus laevis* distal nephron A6 epithelia which express both an apical adenosine receptor of the  $\text{A}_1$  type (coupled to protein kinase C (PKC)) and a basolateral receptor of the  $\text{A}_2$  type (coupled to protein kinase A (PKA)). The untransfected A6 cell line expresses a single NHE type (XNHE) which is restricted to the basolateral membrane and which is activated by PKA.
3. A6 cell lines were generated which express exogenous rat NHE3. Measurements of side-specific  $\text{pH}_i$  recovery from acid loads in the presence of HOE694 (an inhibitor with differential potency towards individual NHE isoforms) detected an apical resistant  $\text{Na}^+ - \text{H}^+$  exchange only in transfected cell lines. The sensitivity of the basolateral NHE to HOE694 was unchanged, suggesting that exogenous NHE3 was restricted to the apical membrane.
4. Stimulation of the apical  $\text{A}_1$  receptor with  $N^6$ -cyclopentyladenosine (CPA) inhibited both apical NHE3 and basolateral XNHE. These effects were mimicked by the addition of the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) and partially prevented by the PKC inhibitor calphostin C which also blocked the effect of PMA.
5. Stimulation of the basolateral  $\text{A}_2$  receptor with CPA inhibited apical NHE3 and stimulated basolateral XNHE. These effects were mimicked by 8-bromo-cAMP and partially prevented by the PKA inhibitor H89 which entirely blocked the effect of 8-bromo-cAMP.
6. In conclusion, CPA inhibits rat NHE3 expressed apically in A6 epithelia via both the apical PKC-coupled  $\text{A}_1$  and the basolateral PKA-coupled  $\text{A}_2$  adenosine receptors.

Adenosine has been identified as a mediator essential for homeostatic regulation of kidney function. Endogenous adenosine generated by adenosine triphosphate (ATP) dephosphorylation (Mi & Jackson, 1995) participates in regulation of renin release and glomerular haemodynamics and influences renal electrolyte transport by regulating a variety of plasma membrane ion channels and transporters (Friedlander & Amiel, 1995; Osswald *et al.* 1997). Increased local production of adenosine which associates with hypernatraemia, ischaemia or administration of nephrotoxic substances reflects an imbalance between energy supply and

energy demand during active transport and has been implicated in acute renal failure (Osswald & Gleiter, 1993*a, b*). Due to the kidney-specific relationship between energy consumption and tubular  $\text{Na}^+$  reabsorption, it is intriguing to consider adenosine regulation of  $\text{Na}^+$  re-absorption (in addition to modulation of renal blood flow and glomerular filtration rate) as an important factor in the pathophysiological control of renal solute and fluid re-absorption.

A few studies only have so far addressed adenosine action on renal  $\text{Na}^+$  transport. From these studies the picture emerges that modulation of renal  $\text{Na}^+$  reabsorption is a

function of the expression of adenosine receptor subtypes on target cells. This is exemplified in primary cultures of rat inner medullary collecting duct cells (Yagil *et al.* 1994) and most likely also in rat medullary thick ascending limbs (Seney & Seikali, 1989) where activation of A<sub>1</sub> receptors inhibit Na<sup>+</sup> reabsorption, and in A6 cells (a cell line derived from *Xenopus laevis* kidney) where activation of A<sub>2</sub> receptors have been shown to stimulate transepithelial Na<sup>+</sup> transport (Lang *et al.* 1985; Casavola *et al.* 1996).

It is generally accepted that the proximal tubule of the kidney reabsorbs the majority of the filtered load of sodium. Current evidence mainly coming from cultured cells but also from native tissue strongly suggests that *NHE3*, which encodes an apical amiloride resistant isoform of the Na<sup>+</sup>-H<sup>+</sup> exchanger (NHE) family, promotes basal- and hormone-stimulated active Na<sup>+</sup> transport in the mammalian proximal tubule (Yun *et al.* 1995; Orłowski & Grinstein, 1997; Wakabayashi *et al.* 1997). In addition, NHE3 is important in HCO<sub>3</sub><sup>-</sup> reabsorption; yet effects of adenosine on NHE3 activity have not been elucidated.

Therefore, in the present study we wished to determine whether adenosine acutely modulates the activity of NHE3. To understand the underlying signalling mechanism(s), experiments were designed to evaluate changes in NHE3 activity in response to either A<sub>1</sub> or A<sub>2</sub> receptor activation. This was accomplished: (i) by stable transfection of cDNA encoding the Na<sup>+</sup>-H<sup>+</sup> exchanger NHE3 (rat isoform) into A6/C1 cells that are devoid of the functional apical Na<sup>+</sup>-H<sup>+</sup> exchanger (Guerra *et al.* 1993; Casavola *et al.* 1996) and are expressing A<sub>1</sub> adenosine receptors on the apical side and A<sub>2</sub> adenosine receptors on the basolateral aspect of the cell surface (Casavola *et al.* 1997), and (ii) by using a series of selective inhibitors of the adenosine effector systems.

The data show that A<sub>1</sub> receptor activation decreases NHE3 activity by a PKC-dependent mechanism and A<sub>2</sub> receptor activation by a PKA-dependent mechanism. Based on the pattern of the pharmacological regulation of the transfected and endogenous Na<sup>+</sup>-H<sup>+</sup> exchanger by PKC and PKA agonists, it is suggested that the endogenous Na<sup>+</sup>-H<sup>+</sup> exchanger (XNHE) in A6/C1 cells is functionally distinct from the ubiquitous NHE1 isoform (Wakabayashi *et al.* 1997), the piscine β-NHE isoform (Borgese *et al.* 1992) and the isoform of the exchanger studied in *Xenopus laevis* oocytes (Busch *et al.* 1995).

## METHODS

### Solutions

Media used in the fluorimetric pH measurements included Na<sup>+</sup> medium composed of (mM): 110 NaCl, 3 KCl, 1 CaCl<sub>2</sub>, 0.5 MgSO<sub>4</sub>, 1 KH<sub>2</sub>PO<sub>4</sub>, 5 glucose and 10 Hepes buffered to pH 7.5 with Tris. TMA medium consisted of (mM): 110 tetramethylammonium chloride (TMAcI), 3 KCl, 1 CaCl<sub>2</sub>, 0.5 MgSO<sub>4</sub>, 1 KH<sub>2</sub>PO<sub>4</sub>, 5 glucose and 10 Hepes buffered to pH 7.5 with Tris. KCl medium contained (mM): 105 KCl, 8 NaCl, 1 CaCl<sub>2</sub>, 0.5 MgSO<sub>4</sub>, 1 KH<sub>2</sub>PO<sub>4</sub>, 5 glucose and 10 Hepes buffered to various pH values for calibration

of the intracellular BCECF (2',7'-bis(carboxymethyl)-5(6)-carboxy-fluorescein-acetoxymethyl ester; Molecular Probes, Eugene, OR, USA) signal.

### Cell culture

Experiments were performed with A6/C1 cells, a subclone of A6-2F3 cells that were selected by ring cloning on the basis of high transepithelial resistance and responsiveness to aldosterone and vasotocin (Verrey, 1994). A6/C1 cell cultures were maintained in 0.8× concentrated DMEM (Life Technologies, Gibco, Basel, Switzerland), containing 25 mM NaHCO<sub>3</sub>, 10% heat-inactivated fetal bovine serum (Life Technologies, Gibco), 50 i.u. ml<sup>-1</sup> penicillin and 50 μg ml<sup>-1</sup> streptomycin (final osmolality: 220–250 mosmol kg<sup>-1</sup>). Cells were incubated in a humidified 95% air–5% CO<sub>2</sub> atmosphere at 28 °C and subcultured weekly by trypsinization using a Ca<sup>2+</sup>-Mg<sup>2+</sup>-free salt solution containing 0.25% (w/v) trypsin and 1 mM EGTA. Cells generally reached confluency between 7 to 8 days after seeding when the culture medium was changed three times a week. Studies on A6/C1 cells were performed between passage 114 to 128.

### Stable transfection and expression of *NHE3* cDNA

Full-length rat *NHE3* cDNA (nucleotides 50–4980) originally obtained by Dr John Orłowski (Montreal, Canada) and Dr Gary Shull (Cincinnati, OH, USA) was subcloned into the mammalian expression plasmid pCMV-5 (gift from Dr David Russel, Dallas, TX, USA) as described previously (Moe *et al.* 1995). A6/C1 cells grown to 20–25% confluence in 35 mm tissue culture dishes were co-transfected with 10 μg *NHE3* cDNA in the pCMV-5 expression plasmid and 0.5 μg of a selection marker termed p3'SS-ΔLacI in 1 ml serum-free culture medium (lacking antibiotics) using the lipophilic reagent polybrene (15 μg (10 μg)<sup>-1</sup> cDNA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Brewer, 1994). The p3'SS-ΔLacI vector which confers resistance to hygromycin B was generated from the eucaryotic *lac* repressor expression vector p3'SS (Stratagene AG, Basel, Switzerland) by excising a *Xba*I–*Eco*RV fragment (nucleotides 1337–2157) in the coding region of the *lacI* gene. Starting from transfection, cells were incubated for 6 h in a humidified 95% air–5% CO<sub>2</sub> atmosphere at 28 °C. Solution was then replaced by serum-free culture medium containing 30% dimethylsulphoxide (DMSO) and cells were allowed to rest for exactly 5 min at room temperature. Thereafter, cells were carefully washed with 2 ml culture medium (composition as given above) and incubated in culture medium in a humidified 95% air–5% CO<sub>2</sub> atmosphere at 28 °C for 24 h. Culture medium was then exchanged and cells were maintained for an additional 12 h in the incubator. To start selection, cells were trypsinized and transferred to 100 mm culture plates, where they were exposed to 450 μg hygromycin B (ml culture medium)<sup>-1</sup> (Calbiochem, Luzern Switzerland) over a period of 2–3 weeks. Hygromycin B-resistant colonies were harvested by ring cloning and were further selected on the basis of expression of NHE3 activity (defined as apical transport activity which is sensitive to inhibition by 8-bromo-cAMP). Cell clones expressing NHE3 activity (A6/*NHE3* cells) were maintained in culture as described above and exposed to hygromycin B during growth in order to maintain selection pressure. As addition of dexamethasone has been shown to accelerate differentiation and to improve the electrical resistance of A6 cell layers (Preston *et al.* 1988), both untransfected and transfected A6 cell lines were grown for 7–8 days in the presence of 1 μM dexamethasone prior to their introduction into experiments.

Experiments on A6/*NHE3* cells were carried out on passages 3 to 36. The data that are presented from A6/*NHE3* cells were obtained from two different cell clones (clones 6 s and 9 s).

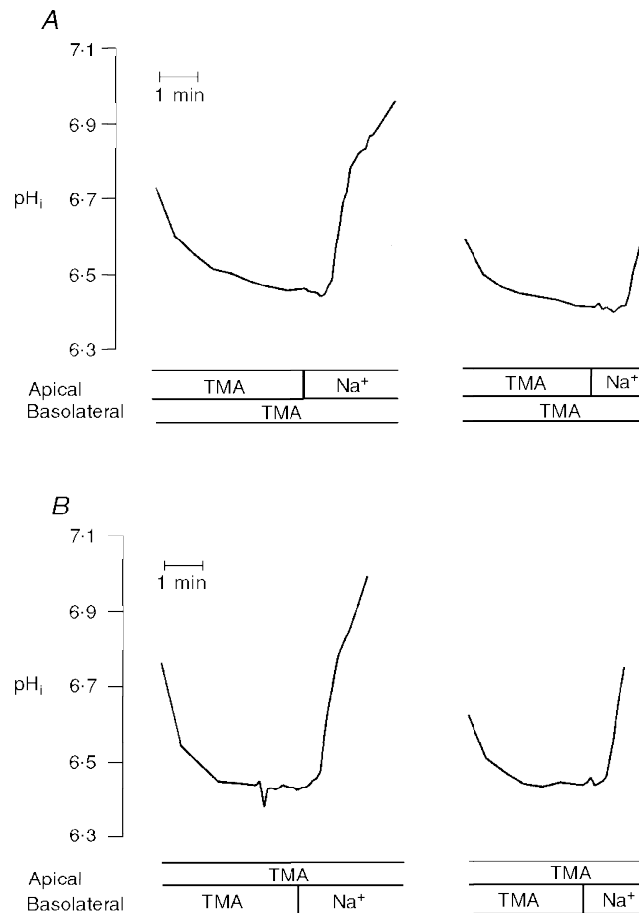
### Measurement of intracellular pH and Na<sup>+</sup>-H<sup>+</sup> exchange activity

For pH experiments, cells were seeded onto collagen-coated coverslips with a 1.5 mm hole punched in the centre covered by a Teflon filter (Millicell-CM, 0.4  $\mu\text{m}$  pore size; Millipore, Eschborn, Germany) as described previously (Helmle-Kolb *et al.* 1997). Cells were incubated for 60 min with 4.2  $\mu\text{M}$  BCECF AM in Na<sup>+</sup> medium containing 50  $\mu\text{M}$  probenecid to minimize possible dye leakage. Dye-loaded cells were mounted into a chamber and perfused continuously at 25 °C with Na<sup>+</sup> medium (at a rate of 0.5 ml min<sup>-1</sup>) on the stage of an inverted microscope (Zeiss IM35) equipped with a Leitz  $\times 50$  water immersion objective. BCECF was excited sequentially by positioning 390–440 nm and 475–490 nm band pass filters in front of a xenon lamp. The excitation light was directed to the cells via a 510 dichroic mirror and emission was collected by a 515–565 nm band pass filter. To minimize dye bleaching a neutral density filter was placed into the excitation light pass. Fluorescence data were recorded every 5 or 10 s by irradiating the cells for 7 ms at each of the wavelengths. Data were

processed and corrected for autofluorescence (measured in cells grown on the filter support but not loaded with BCECF) as previously detailed (Helmle-Kolb *et al.* 1997). Intracellular pH (pH<sub>i</sub>) was estimated from the ratio of BCECF fluorescence calibrated intracellularly by using the K<sup>+</sup>-nigericin approach (Helmle-Kolb *et al.* 1997).

Na<sup>+</sup>-H<sup>+</sup> exchange activity was measured as initial rate of Na<sup>+</sup>-dependent recovery of intracellular pH from an acid load in the absence of HCO<sub>3</sub><sup>-</sup> as described previously (Helmle-Kolb *et al.* 1997). In brief, cells were acid loaded by application of 40 mM NH<sub>4</sub>Cl (in Na<sup>+</sup> medium). Subsequent removal of NH<sub>4</sub>Cl and perfusion with sodium-free TMA medium caused the pH<sub>i</sub> to decrease. Once a stable fluorescence signal was reached, asymmetric expression of Na<sup>+</sup>-H<sup>+</sup> exchanger activity was assessed by measuring Na<sup>+</sup>-dependent alkalinization as a function of Na<sup>+</sup> addition to either the apical or basolateral perfusate.

Effects of agonists on NHE activity were determined by evaluating pH<sub>i</sub> recovery rates of a single cell layer under control conditions and



**Figure 1.** Comparison of rates of Na<sup>+</sup>-H<sup>+</sup> exchange activity in A6/NHE3 cells following repeated acidification of intracellular pH

Na<sup>+</sup>-H<sup>+</sup> exchange activity was studied as Na<sup>+</sup>-dependent recovery of intracellular pH using microspectrofluorometry. For study of the influence of repeated acid loading on the rate of transport activity, confluent cell cultures were pulsed with NH<sub>4</sub>Cl which was then replaced by TMA medium (onset of data presentation). Recovery of pH<sub>i</sub> from an acid load was introduced by addition of Na<sup>+</sup> medium. When rates of alkalinization approached original levels (data not shown), cells were again subjected to a protocol of NH<sub>4</sub>Cl prepulse and withdrawal followed by Na<sup>+</sup> addition. *A*, pH<sub>i</sub> recovery in response to apical Na<sup>+</sup> addition to acid-loaded cells; *B*, pH<sub>i</sub> recovery in response to basolateral Na<sup>+</sup> addition to acid-loaded cells. The tracings are representative of four similar experiments. TMA, perfusion with TMA medium; Na<sup>+</sup>, perfusion with Na<sup>+</sup> medium.

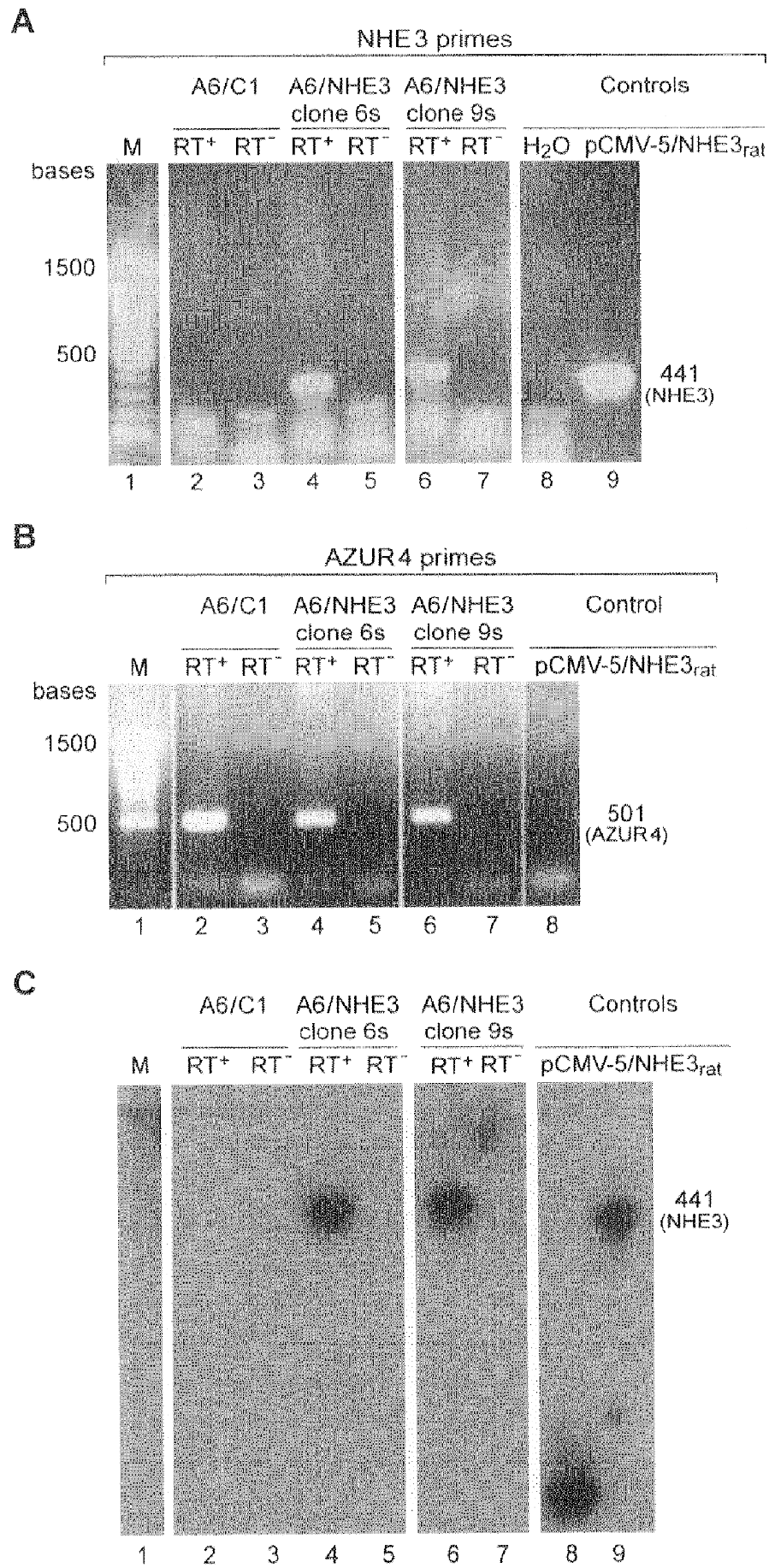


Figure 2. For legend see facing page.

after repeated cell acidification under test conditions. Prior to adopting this protocol, we verified that rates of  $\text{pH}_i$  recovery after consecutive  $\text{NH}_4$  pulses were virtually identical within a cell population when examined from the same starting acid  $\text{pH}_i$  value. An example of a typical control experiment examining the reproducibility  $\text{Na}^+ - \text{H}^+$  exchange activity in either the apical or basolateral membrane of *NHE3*-transfected A6 cells upon repetitive acidification is given in Fig. 1. Following an  $\text{NH}_4$  pulse, cells underwent an acidification and adopted on average an intracellular acid  $\text{pH}$  of  $6.37 \pm 0.016$  ( $n = 15$ , the number of independent experiments from cells of different cell passages) in  $\text{Na}^+$ -free medium. Upon introduction of  $\text{Na}^+$  to either the apical (Fig. 1A) or basolateral cell surface (Fig. 1B) a rapid alkalization ensued (in four independent experiments initial rates of  $\text{pH}_i$  recovery were observed to be  $0.158 \pm 0.012$   $\text{pH units min}^{-1}$  upon apical  $\text{Na}^+$  addition and  $0.236 \pm 0.013$   $\text{pH units min}^{-1}$  upon basolateral  $\text{Na}^+$  addition). Importantly, in pairwise comparisons,  $\text{pH}_i$  recovery rates following a second  $\text{NH}_4$  pulse were indistinguishable from those measured under control conditions (rates of  $\text{pH}_i$  recovery at  $\text{pH}_i$   $6.36 \pm 0.016$  were  $0.167 \pm 0.014$   $\text{pH units min}^{-1}$  upon readdition of apical  $\text{Na}^+$  and  $0.0223 \pm 0.016$   $\text{pH units min}^{-1}$  upon readdition of basolateral  $\text{Na}^+$ ), thus demonstrating the validity of the protocol for comparison of the effect of agonists on basal rate of transport activity. Unless otherwise stated, agonists were introduced into experiments after monitoring of basal transport rates. Cells were then incubated in the presence of agonists for at least 15 min prior to recording their effect on  $\text{Na}^+ - \text{H}^+$  exchange activity. Concentrations of agonist were chosen to be the same as those that have recently been shown to elicit maximal or near-maximal responses in A6/C1 cells (Casavola *et al.* 1997).

#### Total RNA preparation, reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA was extracted from cells grown to confluence on 60 mm culture dishes with TRIzol (Life Technologies, Gibco), following the manufacturer's instructions. Deoxyribonuclease I (Promega GmbH, Mannheim, Germany) treatment was performed to remove any potential genomic DNA contamination.

RT and PCR was carried out essentially as described (Helmle-Kolb *et al.* 1997). In brief, 10  $\mu\text{g}$  of total RNA denatured for 3 min at  $65^\circ\text{C}$  were used for RT with ( $\text{dT}_{15}$ ) primers and avian myeloblastoma virus reverse transcriptase (Promega GmbH). The reaction was carried out in a 20  $\mu\text{l}$  reaction container with 2  $\mu\text{l}$  of 10 $\times$  reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 1% Triton X-100), 5 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{g}$  ( $\text{dT}_{15}$ ) primers, 1 mM of each deoxyribonucleotide (dNTP), 10 i.u. ribonuclease inhibitor (RNasin) and 12.5 i.u. avian myeloblastoma virus reverse transcriptase and incubated at  $42^\circ\text{C}$  for 1 h. To control for cDNA origin, each reaction was performed in parallel with an otherwise identical reaction to which no reverse transcriptase was added.

After RT, 2  $\mu\text{l}$  of the first strand synthesis product were amplified by PCR in a 50  $\mu\text{l}$  reaction using 0.2  $\mu\text{M}$  of each *NHE3* primer in the presence of 2 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, 1.25 i.u. *Taq* polymerase (Promega GmbH), and 5  $\mu\text{l}$  of 10 $\times$  reaction buffer (composition as given above). For control, expression of the amino acid permease-related protein ASUR4 (Spindler *et al.* 1997) was tested. The temperature profile for PCR reaction was: hot start ( $95^\circ\text{C}$  for 2 min); followed by 40 cycles consisting of denaturing at  $95^\circ\text{C}$  (for 40 s), annealing at  $48^\circ\text{C}$  (40 s), and extension at  $72^\circ\text{C}$  (for 40 s); final extension was at  $72^\circ\text{C}$  (for 10 min). PCR products were separated on 1.5% agarose gels stained with ethidium bromide. Bands of the expected sizes were visualized under UV light.

*NHE3* primers used for PCR reaction encode a sequence of 441 bp in the cytoplasmic tail of *NHE3* (starting from amino acid (aa) 684 until aa 837) Primer sequences were: 5'-ACAGAAGCGGAGGAA-TAG-3' (sense strand) and 5'-CATGTGTGTGGACTCAGG-3' (antisense strand).

Primers sets for ASUR4 detect a 501 bp sequence (aa 244 to aa 410). Sequences were: 5'-GTCCTGGCATTGTACAGT-3' (sense strand) and 5'-CAGGGCTACGCAAAGCCA-3' (antisense strand).

#### Southern blot analysis of PCR products

For Southern blot analysis of PCR products, gels were denatured (in 0.5 M NaOH, 1.5 M NaCl) at room temperature for 30 s, vacuum transferred (Bio-Rad, Glattbrugg, Switzerland) to a nylon filter (Pall, Biotyne B transfer membrane), and UV crosslinked at 312 nm for 5 min. The nylon filter was pre-hybridized for 30 min in hybridization solution (containing 5 $\times$  Denhardt's reagent (containing per 500 ml: 5 g Ficoll (type 400: Pharmacia), 5 g polyvinylpyrrolidone and 5 g bovine serum albumin (fraction V, Sigma-Aldrich Chemie), made up to 500 ml with  $\text{H}_2\text{O}$ ), 5 $\times$  saline-sodium citrate buffer (SSC), 0.5% SDS, and 100  $\mu\text{g ml}^{-1}$  denatured herring sperm DNA) at  $62^\circ\text{C}$ . Hybridization was carried out for 5 h using 10 ml of hybridization solution containing 25  $\mu\text{l}$  of [ $^{32}\text{P}$ ]-labelled 3 kb rat *NHE3* cDNA ( $10^{-6}$  c.p.m.  $\text{ml}^{-1}$ ) (the 3 kb *NHE3* cDNA fragment was obtained from the pMVC5/*NHE3*<sub>rat</sub> construct by digestion with the restriction enzyme *Bam*HI (Biofinex) followed by electrophoresis on a 1.5% agarose gel and extraction with the gene clean II kit (Bio 101, 1070 Joshua Weg, Vista, CA, USA)). The probes obtained were labelled using the commercially available oligolabelling kit from Pharmacia (Freiburg im Breisgau, Germany). The blots were washed twice for 5 min at room temperature in 2 $\times$  SSC and 0.1% SDS and twice for 30 min at  $65^\circ\text{C}$  in 0.5 $\times$  SSC and 0.1% SDS, and then analysed by autoradiography.

#### Other methods

Monolayer resistance was measured on A6 cells grown on collagen-coated polycarbonate filter rings (Transwell, 0.4  $\mu\text{m}$  pore size,

#### Figure 2. Analysis of transcripts for *NHE3* in A6/C1 and A6/*NHE3* cells (clone 6 s and clone 9 s)

Total RNA was extracted from A6/C1 or A6/*NHE3* cells and used to detect message for *NHE3* (panel A) or AZUR4 (positive control; panel B) with RT-PCR. Southern blot hybridization (\*, see below) to [ $^{32}\text{P}$ ]-labelled, 3 kb *NHE3* cDNA was then used to determine the identity of PCR products obtained with the *NHE3* primer set (panel C). RT<sup>+</sup>, RT reactions performed in the presence of reverse transcriptase; RT<sup>-</sup>, RT reactions performed in the absence of reverse transcriptase. pCMV-5/*NHE3*<sub>rat</sub>, PCR reaction performed with the linearized expression plasmid containing the full sequence of rat *NHE3*; M, molecular mass marker (100 bp ladder from Life Technologies, Gibco). \*Note that the band in lane 8 of the Southern blot represents hybridization of pCMV-5/*NHE3* to 3 kb *NHE3* cDNA, because PCR probes were run on a single agarose gel with an upper and lower slit row.

Table 1. Concentration response profiles for HOE694 on Na<sup>+</sup>-H<sup>+</sup> exchange activities in A6/C1 and A6/NHE3 cells

Cell type	NHE activity	[HOE694]			
		5 × 10 <sup>-5</sup> M	7.5 × 10 <sup>-5</sup> M	10 <sup>-4</sup> M	10 <sup>-3</sup> M
A6/C1	Basolateral	57.6 ± 4.3*†§ (4)	69.0 ± 6.2*†§ (3)	96.8 ± 3.2*†§ (3)	—
A6/NHE3, clone 6s	Apical	28.0 ± 3.7*† (5)	41.3 ± 4.5*† (3)	48.7 ± 4.3*† (4)	94.5 ± 3.3* (4)
	Basolateral	59.7 ± 5.3* (4)	64.9 ± 1.8* (4)	85.5 ± 1.8* (3)	—
A6/NHE3, clone 9s	Apical	33.4 ± 2.5*† (5)	39.7 ± 6.2*† (4)	46.1 ± 5.7*† (7)	—
	Basolateral	57.8 ± 4.8* (4)	74.1 ± 5.7* (5)	89.8 ± 4.6* (3)	—

Values are the mean ± s.e.m. of the NHE activity inhibition (%) with the number of experiments performed under identical conditions (*n*) given in parentheses. Confluent cell cultures of A6/C1 and A6/NHE3 cells were examined for Na<sup>+</sup>-dependent recovery of pH<sub>i</sub> from an acid load as described in Fig. 3. Effects of HOE694 were studied after repetitive acidification of cell cultures to control acid pH<sub>i</sub> values. Data represent the initial decrease of pH<sub>i</sub> recovery rates resulting from a 2 min exposure of either the apical or basolateral cell surface to indicated concentrations of HOE694. \*Significant *vs.* control, †significant *vs.* basolateral effect of HOE694, ‡significant compared with apical NHE inhibition in clone 6s and §significant compared with apical NHE inhibition in clone 9s.

4.7 cm<sup>2</sup>, CorningCostar, Cambridge, MA, USA) in a modified Ussing chamber with an automatic voltage-clamp apparatus connected to a dual-channel recorder as described (Verrey, 1994). For experiments culture medium was replaced by Na<sup>+</sup> medium (composition as given above) and measurements were carried out at room temperature.

#### Data presentation

Results are presented as means ± standard error (s.e.m). Statistical significance was established by analysis of variance (ANOVA). A probability (*P*) < 0.05 was considered statistically significant.

## RESULTS

### Functional expression of NHE3 in transfected cells

Three complementary approaches were used to confirm NHE3 expression in transfected A6 cells (A6/NHE3). First, RT-PCR of the NHE3 region corresponding to the C terminus of the protein served to verify that the NHE3 transcript was expressed in hygromycin- (selection marker) resistant cell clones. Second, inhibitor sensitivity (HOE694, (3-methylsulphonyl-4-piperidinobenzoyl)guanidine methane-sulphonate), kindly provided by Dr H. Lang, (Hoechst, Germany) was used to document that endogenous and transfected NHE activities can be separated pharmacologically. Third, analysis of cAMP responses was performed to establish that endogenous and transfected NHE activities can be separated functionally.

Figure 2 shows the results of a RT-PCR analysis on expression of NHE3 transcripts. As shown in Fig. 2A, primers specific for rat NHE3 detected a product of 441 bp

when PCR reactions were carried out on cDNA templates obtained by reverse transcription of RNA samples from NHE3 transfectants (clone 6 s and clone 9 s; Fig. 2A, lanes 4 and 6). A product of similar size was obtained with the linearized pCMV-5 expression plasmid containing the full length NHE3 cDNA (Fig. 2A, lane 9). No discernable products were detected when cDNA obtained by reverse transcription of A6/C1 cell RNA (parent cell line) served as a template (Fig. 2A, lane 2). Primers specific for AZUR4 (Spindler *et al.* 1997) resulted in a product of 501 bp with RNA from A6/C1 and A6/NHE3 cells (Fig. 2B), thereby confirming the selected RT-PCR reaction sequence. Samples analysed in the absence of reverse transcriptase prevented the appearance of the 441 bp and 501 bp RT-PCR products, ruling out contamination of samples with genomic DNA. That A6/NHE3 transfectants are indeed expressing the transcript encoding NHE3 was documented by Southern blot analysis. As shown in Fig. 2C, the NHE3 cDNA probe hybridized with cDNA obtained from A6/NHE3 cells, but not from A6/C1 cells.

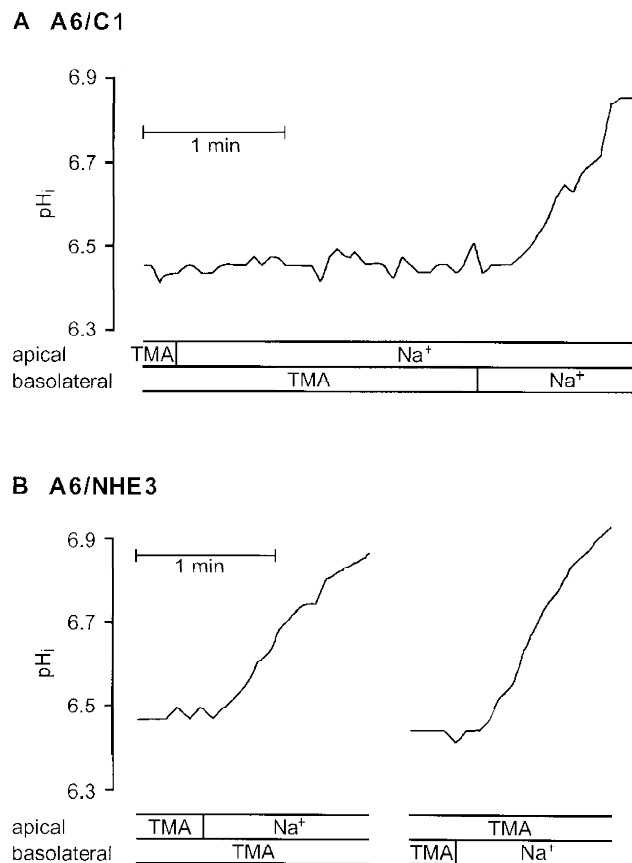
Figure 3 illustrates experiments on the time course of Na<sup>+</sup>-dependent pH<sub>i</sub> recovery from an acid load in A6/C1 and A6/NHE3 cells as a function of apical or basolateral Na<sup>+</sup> addition. In agreement with earlier observations (Guerra *et al.* 1993) only addition of basolateral Na<sup>+</sup> initiated pH<sub>i</sub> recovery in A6/C1 cells (0.192 ± 0.039 pH units min<sup>-1</sup>; *n* = 20) (Fig. 3A). In A6/NHE3 cells substantial pH<sub>i</sub> recovery rates were observed upon addition of both apical Na<sup>+</sup> (0.167 ± 0.014 pH units min<sup>-1</sup>; *n* = 48) (Fig. 3B, left) and basolateral Na<sup>+</sup> (0.223 ± 0.0164 pH units min<sup>-1</sup>; *n* = 46)

(Fig. 3B, right). As monolayer resistance was observed to be  $7.16 \pm 0.72$  and  $6.82 \pm 0.22 \text{ k}\Omega \text{ cm}^2$  in A6/C1 and A6/NHE3 cells, respectively, the occurrence of  $\text{pH}_i$  transients in response to apical  $\text{Na}^+$  addition to acid-loaded A6/NHE3 cells is unlikely to arise from paracellular ion leak.

Although preceding experiments suggest that NHE3 is present in A6/NHE3 cells, they do not define the contribution of NHE3 to apical and/or basolateral  $\text{Na}^+-\text{H}^+$  exchange activity. To assess the fraction of  $\text{Na}^+-\text{H}^+$  exchange mediated by NHE3 we have used the NHE specific inhibitor, HOE694, which inhibits  $\text{Na}^+-\text{H}^+$  exchanger isoforms at widely differing concentrations (Noël & Pouyssegur, 1995), thus being capable of distinguishing between 'resistant' (NHE3) and sensitive isoforms (NHE1 and NHE2) of the  $\text{Na}^+-\text{H}^+$  exchanger. As shown in Table 1, basolateral  $\text{pH}_i$  recovery of both A6/C1 and A6/NHE3 cells was progressively inhibited with increasing concentrations of HOE694, with 85% inhibition occurring at  $10^{-4} \text{ M}$  HOE694

in NHE3 transfectants. Apical  $\text{pH}_i$  recovery in the A6/NHE3 cells was found to be less sensitive to HOE694. Of note, there was no significant difference between inhibition profiles of both apical and basolateral NHE activities in clone 6 s and clone 9 s and basolateral NHE activities in untransfected and transfected cell lines (Table 1). Together, inhibition data are indicative of a preferential distribution of NHE3 on the apical side of A6/NHE3 cells. Whether  $\text{Na}^+$ -dependent  $\text{pH}_i$  recovery in the apical membrane is consequent to expression of NHE3 alone or a combination of NHE3 and endogenous  $\text{Na}^+-\text{H}^+$  exchanger (XNHE) cannot be determined by the present findings, since the difference in inhibitor sensitivity of apical and basolateral  $\text{Na}^+-\text{H}^+$  exchange activity was so small.

Several lines of evidence suggest that the behaviour of the A6 cell  $\text{Na}^+-\text{H}^+$  exchanger and NHE3 to PKA activation is likely to be diametrically opposed, the former is stimulated (Guerra *et al.* 1993) while the latter is inhibited (Helmle-Kolb *et al.* 1990; Moe *et al.* 1995; Cabado *et al.* 1996). This

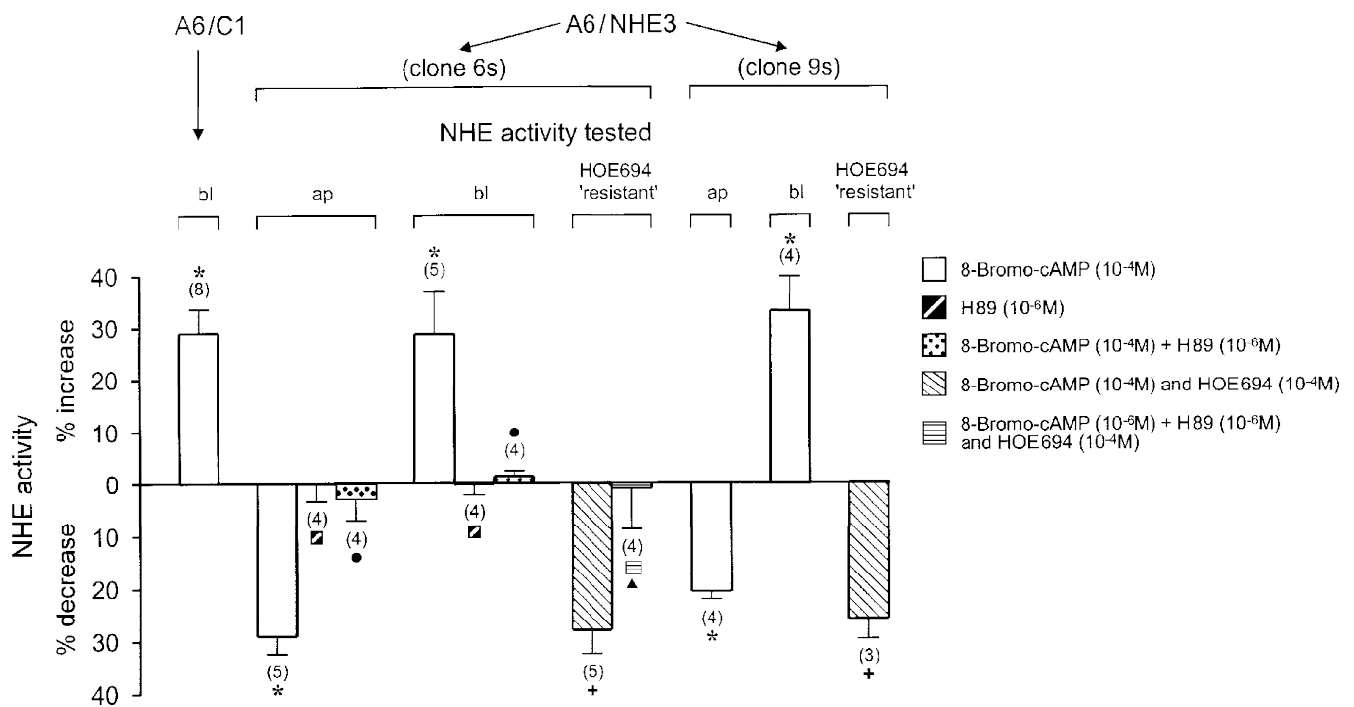


**Figure 3.  $\text{Na}^+$  dependence and polarity of  $\text{pH}_i$  recovery in acid-loaded A6/C1 and A6/NHE3 cells**  
 Confluent cell cultures of A6/C1 or A6/NHE3 cells on filter support were examined for  $\text{pH}_i$  change by microspectrofluorometry using the dye BCECF as detailed in Methods. Cells in  $\text{Na}^+$  medium were pulsed with  $40 \text{ mM NH}_4\text{Cl}$  and then perfused with  $\text{Na}^+$ -free medium (TMA). Recovery of  $\text{pH}_i$  from an acid load was next induced by re-introduction of  $\text{Na}^+$  medium ( $\text{Na}^+$ ) to either the apical or the basolateral cell surface. Fluorescence ratio traces from A6/C1 cells (A) and from A6/NHE3 cells (B), representative of at least four independent experiments.

prompted us to validate the fraction of contribution of NHE3 to A6 cell  $\text{Na}^+-\text{H}^+$  exchange activity by measuring the rates of  $\text{Na}^+$ -dependent  $\text{pH}_i$  recovery in acid-loaded cells pretreated with or without 8-bromo-cAMP. As shown in Fig. 4, 8-bromo-cAMP ( $10^{-4}$  M for 15 min) significantly stimulated basolateral NHE activity in A6/C1 cells (by 29%) as well as in A6/NHE3 cells (by 29% in clone 6 s and 33% in clone 9 s), but decreased NHE activity located on the apical side of A6/NHE3 cells (by 29% in clone 6 s and by 21% in clone 9 s). Stimulation of basolateral NHE activity by 8-bromo-cAMP was not significantly different between untransfected and transfected cell lines. Likewise, the percentage of inhibition of apical NHE activity was not significantly different between transfected cell cultures (clones 6 s and 9 s), although 8-bromo-cAMP inhibited apical activity in clone 6 s slightly more. As expected, 8-bromo-cAMP-induced changes of NHE activities in A6/NHE3 cells (clone 6 s) were prevented by the PKA antagonist,

H89, which when tested alone had no influence on rates of  $\text{Na}^+-\text{H}^+$  exchange. This indicated that the stimulatory and inhibitory actions of 8-bromo-cAMP were mediated by PKA. Importantly, in experiments evaluating the effect of 8-bromo-cAMP in the presence of HOE694 concentrations that completely blocked NHE activity located in the basolateral membrane of A6 cells (see Table 1), 8-bromo-cAMP-evoked responses of apical  $\text{Na}^+-\text{H}^+$  exchanger (clones 6 s and 9 s) were barely distinguishable from those obtained in the absence of the inhibitor. These results imply that endogenous XNHE activity is either absent from the apical side or completely unresponsive to PKA agonists when expressed in the apical membrane context of A6/NHE3 cells.

As adenosine induces stimulation of PKA and PKC in A6 cells, we proceeded to test the sensitivity of the transfected  $\text{Na}^+-\text{H}^+$  exchanger NHE3 to PKC activation. To this end,



**Figure 4. Modulation of NHE activity by a PKA agonist/antagonist**

$\text{Na}^+-\text{H}^+$  exchange activity was assessed as initial rates of HOE694 inhibitable  $\text{pH}_i$  recovery of A6 cell cultures from an acid load as described in Fig. 3. To test the effect of 8-bromo-cAMP (PKA agonist) and/or H89 (PKA antagonist), cell cultures were repetitively acidified to the same starting acid  $\text{pH}_i$  value, and  $\text{pH}_i$  recovery rates were observed either in the absence or presence of pharmacological agents added to the apical and basolateral cell surface. In all experiments, cells were exposed to different agents for 15 min prior to evaluating their effect on  $\text{Na}^+-\text{H}^+$  exchange activity in the absence or presence of HOE694 that was included for a 2 min period in the apical perfusate. The number of experiments performed under identical experimental conditions is given in parentheses. ap, the change (expressed in %) of the activity of apical  $\text{Na}^+-\text{H}^+$  exchanger in response to pharmacological agents; bl, the corresponding change (expressed in %) of basolateral  $\text{Na}^+-\text{H}^+$  exchanger; HOE694 'resistant', agonist-induced net change (expressed in %) of apical transport activity which was calculated as difference between the sum of  $\text{Na}^+$ -dependent rate of  $\text{pH}_i$  recovery measured in the presence of agonists and  $10^{-4}$  M apical HOE694 and  $10^{-4}$  M apical HOE694 alone. \* Significant *vs.* control; + significant *vs.* HOE694; • significant *vs.* 8-bromo-cAMP; ▲ significant *vs.* 8-bromo-cAMP plus HOE694.

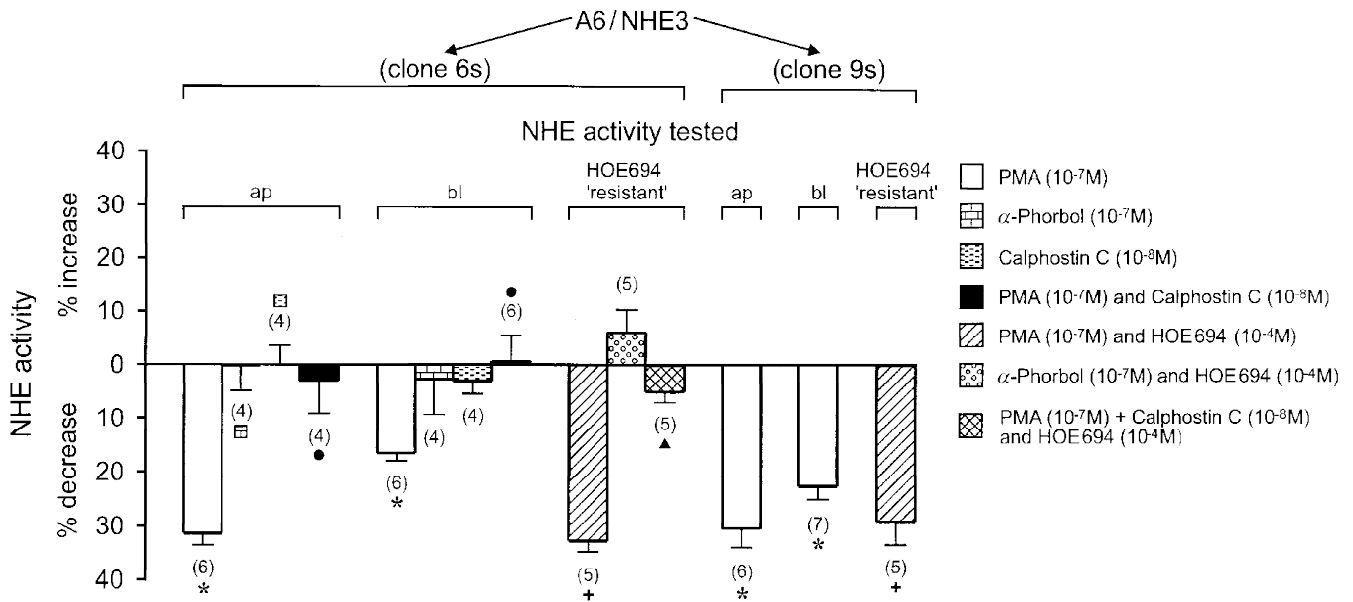


Na<sup>+</sup>-dependent pH<sub>i</sub> recovery rates in A6/NHE3 cells were analysed prior to and following a 15 min incubation with the PKC agonist, phorbol 12-myristate 13-acetate (PMA). In these and further experiments we abstained from comparison of A6/NHE3 data with A6/C1 cell data, because transfection, as judged from the preceding results, did not noticeably alter basolateral transport characteristics. As illustrated in Fig. 5, PMA (10<sup>-7</sup> M) inhibited Na<sup>+</sup>-H<sup>+</sup> exchange activity both in the apical and basolateral membrane of A6/NHE3 cells. The inert analogue 4α-phorbol had no effect on either of the transport activities (clone 6 s). Calphostin C, a specific inhibitor of PKC, effectively prevented the inhibitory action of PMA (clone 6 s), suggesting that downregulation of NHE activities in A6/NHE3 cells was indeed due to activation of PKC. Again, the presence of HOE694, even though it partially inhibited apical NHE activity, did not alter the fraction of Na<sup>+</sup>-H<sup>+</sup> exchange activity inhibited by PMA (clones 6 s and 9 s). Likewise, HOE694 did not influence typical responses of apical Na<sup>+</sup>-H<sup>+</sup> exchanger (clone 6 s) to PMA and calphostin C. Since regulation of NHE3 in A6 cells largely resembles protein kinase regulation of the renal brush border membrane Na<sup>+</sup>-H<sup>+</sup> exchanger in other cell systems (Helmle-Kolb *et al.* 1990, 1997; Levine *et al.* 1993; Azarani *et al.* 1995; Cabado *et al.* 1996), the present data are

interpreted to indicate that the A6 cell model is suited for study of the mechanism of NHE3 modulation by biological stimuli acting through PKA and PKC.

**Influence of adenosine on the Na<sup>+</sup>-H<sup>+</sup> exchanger activity in A6/NHE3 cells**

Distinct adenosine receptor subtypes have previously been found to be expressed in A6/C1 cells: A<sub>1</sub> receptors functionally coupled to the phospholipase C-PKC effector system were shown to reside on the apical cell surface, whereas A<sub>2</sub> receptors coupled to the adenylate cyclase-PKA effector system were found to be expressed on the basolateral side (Casavola *et al.* 1997). Based on these earlier observations, addition of either apical or basolateral adenosine (in the absence or presence of PKC or PKA antagonists) would therefore provide a useful means of determining whether activation of A<sub>1</sub> or A<sub>2</sub> receptors is associated with NHE3 regulation. As preceding results indicate that all NHE regulation in clones 6 s and 9 s is virtually identical, experiments testing adenosine effects were conducted on clone 6 s only. As can be seen from Fig. 6A, apical addition of the metabolically stable adenosine analogue N<sup>6</sup>-cyclopentyladenosine (CPA, 10<sup>-6</sup> M for 15 min), significantly reduced NHE activity located in the apical membrane (by 31 %) and in the basolateral membrane

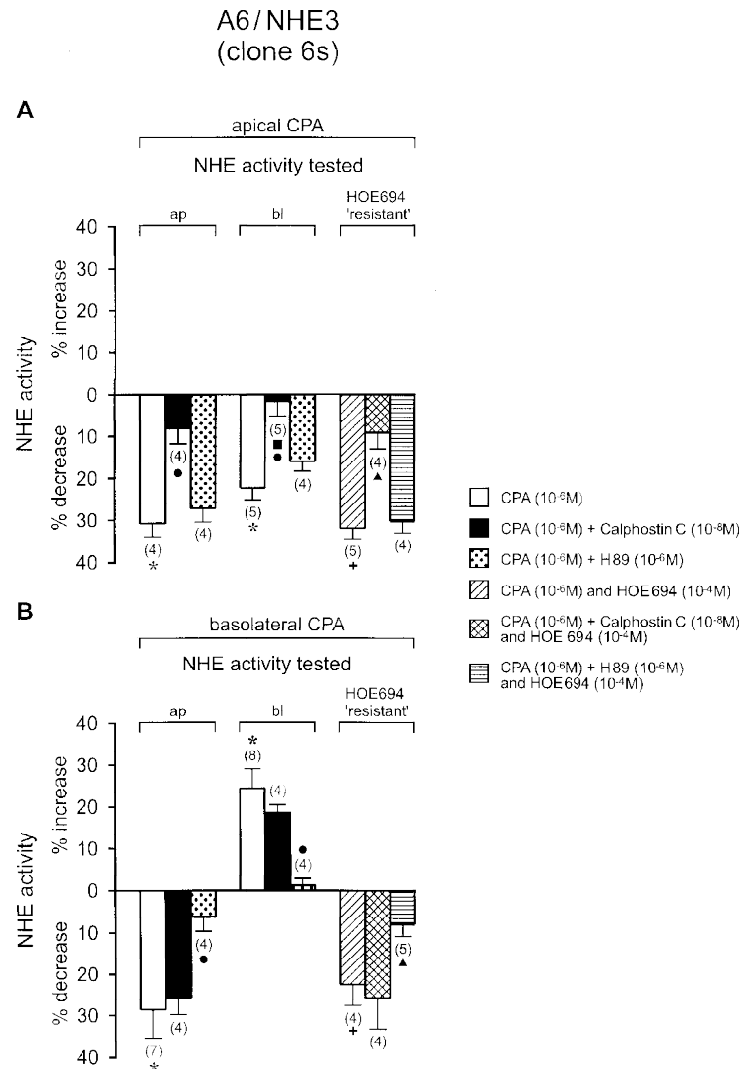


**Figure 5. Modulation of NHE activities by a PKC agonist/antagonist**

The effect of PMA (PKC agonist) and/or calphostin C (PKC antagonist) on Na<sup>+</sup>-H<sup>+</sup> exchange activities of confluent A6/NHE3 cells was tested as indicated in the legend to Fig. 4. Exposure to different agents (which were included in the apical and basolateral perfusate) was for 15 min, except for HOE694 which was added to the apical perfusate 2 min prior to evaluating the influence of different agents on Na<sup>+</sup>-dependent rate of alkalinization. The number of experiments performed under identical experimental conditions is given in parentheses. ap, change (expressed in %) of the activity of apical Na<sup>+</sup>-H<sup>+</sup> exchanger in response to pharmacological agents; bl, corresponding change (expressed in %) of basolateral Na<sup>+</sup>-H<sup>+</sup> exchanger; HOE694 'resistant', change (expressed in %) of apical transport rates in response to pharmacological agents and HOE694 when normalized to inhibition of 10<sup>-4</sup> M HOE694 alone. \* Significant vs. control; + significant vs. HOE694; • significant vs. PMA; ▲ significant vs. PMA plus HOE694.

(by 22%). Calphostin C abrogated CPA inhibition of apical NHE activity only partially, but evoked a significant reduction of the CPA response of basolateral  $\text{Na}^+-\text{H}^+$  exchanger. H89 had no significant effect on transport responses elicited by apical CPA. To verify that CPA indeed inactivated NHE3, experiments were also conducted in the presence of HOE694. As shown in Fig. 6A, HOE694 concentrations that specifically inactivated XNHE activity had no effect on CPA-induced downregulation of apical

$\text{Na}^+-\text{H}^+$  exchanger. As is also shown, calphostin C prevented CPA inhibition of apical  $\text{Na}^+-\text{H}^+$  exchange activity with a similar efficacy as in the corresponding experiments performed in the absence of the inhibitor. Furthermore, as observed in the experiments above, H89 was unable to prevent inhibition of apical  $\text{Na}^+-\text{H}^+$  exchanger by CPA. Of note, the  $\text{A}_1$  receptor blocker CPX (1,3-dipropyl-8-cyclopentylxanthine) inhibited CPA-induced downregulation of apical  $\text{Na}^+-\text{H}^+$  exchanger effectively without appreciably



**Figure 6.** CPA effects on NHE activities of A6/NHE3 cells in clone 6s, in the absence and presence of PKC or PKA antagonists

*A*, effect of apical CPA (adenosine analogue) on rate of  $\text{Na}^+-\text{H}^+$  exchanger. *B*, effect of basolateral CPA on rate of  $\text{Na}^+-\text{H}^+$  exchanger. NHE activities were observed under control and test conditions as indicated in Fig. 4. Except for CPA, agents were applied to the apical and basolateral aspect of the cells. Changes in rate of NHE activities were observed 15 min after application of pharmacological agents in the presence or absence of HOE694 (which was added to the apical perfusate 2 min prior to initiation of  $\text{Na}^+$ -dependent cell alkalization). The number of experiments performed under identical experimental conditions is given in parentheses. ap, change (expressed in %) of the activity of apical  $\text{Na}^+-\text{H}^+$  exchanger in response to pharmacological agents; bl, corresponding change (expressed in %) of basolateral  $\text{Na}^+-\text{H}^+$  exchanger; HOE694 'resistant', change (expressed in %) of apical transport rates in response to pharmacological agents and HOE694 when normalized to inhibition of  $10^{-4}\text{M}$  HOE694 alone. \* Significant vs. control; + significant vs. HOE694; • significant vs. CPA; ▲ significant vs. CPA plus HOE694.

altering basal NHE3 activity (compared with controls, transport rates were altered by  $0.3 \pm 3.7\%$ ,  $n = 5$ , in the presence of CPA and CPX, and by  $5.1 \pm 3.4\%$ ,  $n = 4$ , in the presence of CPX alone).

Figure 6B illustrates the effect of basolateral CPA on the  $\text{Na}^+-\text{H}^+$  exchanger. Treatment of cells with basolateral CPA ( $10^{-6}$  M for 15 min) significantly decreased apical NHE activity (by 29%) but caused an increase of basolateral XNHE activity (by 24%). As expected, H89 almost completely eliminated CPA inhibition of apical  $\text{Na}^+-\text{H}^+$  exchanger and effectively reduced CPA activation of basolateral XNHE. In contrast, calphostin C was unable to negate transport responses following basolateral CPA addition. Of note, treatment with HOE694 had no influence on downregulation of apical NHE activity by CPA (in the absence or presence of calphostin C). Likewise, there was no significant change in the degree of the reversal of CPA actions by H89 when experiments were done in the presence of HOE694. Together, these results indicate that the  $\text{A}_1$  receptor action on NHE3 activity in A6/NHE3 cells involves PKC, while  $\text{A}_2$  receptor action involves PKA.

## DISCUSSION

The aim of the present study was to study regulation of the  $\text{Na}^+-\text{H}^+$  exchanger NHE3 by the adenosine analogue  $N^6$ -cyclopentyladenosine (CPA). Since future studies aim to identify structural elements involved in the molecular mechanism of NHE3 regulation by CPA, the present studies were carried out in A6/C1 cells that lack endogenous expression of NHE3 (see Fig. 2A and C, and Guerra *et al.* 1993; Casavola *et al.* 1997) and are able to activate the PKC and the PKA pathway in response to  $\text{A}_1$  or  $\text{A}_2$  receptor stimulation, respectively (see Figs 4 and 5, and Casavola *et al.* 1997).

The present functional data provide evidence that NHE3 is preferentially routed to the apical membrane of A6/C1 cells. This site of expression is in agreement with the reported location of NHE3 in the renal epithelia, including the proximal tubule, and the thin and thick ascending limbs of the loop of Henle of the rat kidney (Amemiya *et al.* 1995; Biemesderfer *et al.* 1997). It is also in agreement with the location of the transporter in cultured renal epithelial cells expressing either endogenous or transfected NHE3 activity, such as opossum kidney (OK) cells (Amemiya *et al.* 1995) and Madin–Darby canine kidney (MDCK) cells (Helmle-Kolb *et al.* 1997). That NHE3 is the major contributor to apical NHE activity in transfected A6/C1 cells was judged by the effect of HOE694 and 8-bromo-cAMP. The concentration of HOE694 required to inhibit ~50% of apical NHE activity was between 100 and 500  $\mu\text{M}$ , and was similar to the concentration required for half-maximal inhibition of NHE3 in other cell systems ( $K_{0.5} = 650 \mu\text{M}$ ) (Noël & Pouyssegur, 1995). Interestingly, although the sensitivity of apical and basolateral  $\text{Na}^+-\text{H}^+$  exchanger to

HOE694 was significantly different at all concentrations tested, the potency of HOE694 to inhibit basolateral XNHE in A6/NHE3 cells and A6/C1 cells was rather weak. This finding could be interpreted to indicate that introduction of NHE3 into A6 cells may lead to random targeting of the transfected  $\text{Na}^+-\text{H}^+$  exchanger to the apical and basolateral cell surface. This possibility, however, is very unlikely, because inhibition of basolateral  $\text{Na}^+-\text{H}^+$  exchanger in A6/NHE3 was indistinguishable from that in A6/C1 cells (parent cell line). Furthermore, in the experiments examining PKA modulation of apical and basolateral  $\text{Na}^+-\text{H}^+$  exchanger in A6/NHE3 cells we have shown that the PKA agonist 8-bromo-cAMP selectively inhibited apical NHE activity, whereas it stimulated basolateral NHE activity. Although consistent with earlier findings in OK cells, where NHE3 mediates apical transport (Helmle-Kolb *et al.* 1990), the cellular context has to be considered when accounting for the distinctive regulation of NHE3 by PKA (Wakabayashi *et al.* 1997), since responsiveness of NHE3 to agonists that elevate cAMP has been shown to depend largely on expression of cell-specific co-factors (Weinman *et al.* 1993; Yun *et al.* 1997) and most probably requires direct phosphorylation of the transport protein (Moe *et al.* 1995; Kurashima *et al.* 1997; Orłowski & Grinstein, 1997). As regards activation of basolateral XNHE activity by PKA, findings in A6/C1 and A6/NHE3 cells are comparable to PKA modulation of the trout  $\beta$ -NHE isoform (Borgese *et al.* 1992) and the exchanger from *Xenopus laevis* oocytes (XL-NHE) (Busch *et al.* 1995), but distinct from that reported for NHE1 (Noël & Pouyssegur, 1995). PCR amplification and sequencing of the A6 cell exchanger revealed that XNHE is nearly identical to the XL-NHE in *Xenopus laevis* oocytes (Busch, 1997; F. Di Sole & F. Verrey, unpublished observations), thereby highlighting the possibility that amphibian NHEs are probably a distinct class of  $\text{Na}^+-\text{H}^+$  exchanger.

In the present study we have shown that activation of PKC by PMA resulted in an inactivation of both apical NHE3 (Fig. 5) and basolateral XNHE activity in transfected (Fig. 5) and untransfected A6 cell cultures (data not shown). Despite earlier controversy, it is now generally accepted that PMA and/or PKC inhibit endogenous as well as transfected NHE3 activity in a variety of cells, including OK cells (Helmle-Kolb *et al.* 1990), NHE3 transfected Chinese hamster ovary cells (AP-1) (Kandasamy *et al.* 1995) and NHE3 transfected Chinese hamster lung fibroblasts (PS120) (Yip *et al.* 1997). Somewhat unexpected is the type of control of basolateral XNHE activity, since PKC has been reported to upregulate the activity of XL-NHE, which as stated above is highly homologous to XNHE. Although direct proof is as yet not available, a decrease in basolateral membrane area, as recently observed in A6 cells in response to PKC stimulation (Beron *et al.* 1997), could explain the overall inhibitory effect of PMA on basolateral XNHE activity. Alternatively, kidney-specific splicing of the

amphibian  $\text{Na}^+-\text{H}^+$  exchanger may be responsible for the ability of the exchanger to positively or negatively respond to PKC stimulation.

The results of this study provide direct evidence that the activity of NHE3 can be regulated by adenosine. Most notable, our studies suggest that stimulation of  $\text{A}_1$  receptors (linked to activation of PKC) and  $\text{A}_2$  receptors (linked to activation of PKA) produce qualitatively similar effects on NHE3 activity (inhibition of transport rate). That activation of  $\text{A}_1$  and  $\text{A}_2$  receptors contributes to NHE3 regulation is concluded from the following observations: (i) the ability of the  $\text{A}_1$  receptor blocker CPX to antagonize NHE3 modulation following apical adenosine addition, (ii) the ability of the PKA antagonist H89 to abrogate basolaterally evoked NHE regulation by adenosine in A6/NHE3 and A6/C1 cells, and finally, our recent findings that the  $\text{A}_2$  receptor blocker CSC (8-(3-chlorostyryl)caffeine) completely prevented adenosine effects in A6/C1 cells following basolateral nucleoside addition (Casavola *et al.* 1997).

However, as opposed to modulation of XNHE,  $\text{A}_1$  and  $\text{A}_2$  receptor activation of the PKC and PKA pathway appears not to account fully for the overall effect of adenosine on NHE3 activity. Inhibition of PKC by calphostin C prevented inactivation of NHE3 following apical adenosine only partially, and PKA antagonism by H89 did not fully avert transport responses stimulated by basolateral adenosine addition. This suggests that an, as yet unknown, mechanism participates in adenosine regulation of apical NHE3 activity in A6/NHE3 cells. Preliminary experiments in our laboratory indicate that stimulation of  $\text{A}_3$  receptors induces an elevation of intracellular  $[\text{Ca}^{2+}]_i$  in A6/C1 cells. Interestingly, epithelial isoforms of the  $\text{Na}^+-\text{H}^+$  exchanger (NHE2 and NHE3) have been suggested to become inhibited when  $[\text{Ca}^{2+}]_i$  increases (Cohen *et al.* 1990; Burns *et al.* 1991). Whether an increase in  $[\text{Ca}^{2+}]_i$  participates in adenosine modulation of apical NHE3 activity remains to be established.

Finally, our data confirm our previous observations (Casavola *et al.* 1997) that XNHE is under the control of adenosine. This points to a role of adenosine in acute regulation of intracellular pH, serving as a starting signal to change renal electrolyte excretion. In view of this argument, it is worth mentioning that  $\text{K}^+$  excretion has been observed to increase as intracellular pH rises (Gekle *et al.* 1996).

In conclusion, transfected A6/C1 cultures allowed us to confirm that adenosine decreases the activity of the renal brush border membrane  $\text{Na}^+-\text{H}^+$  exchanger NHE3 with PKC playing a role in transport responses to  $\text{A}_1$  receptor activation and PKA being involved in transport responses effected by  $\text{A}_2$  receptor stimulation. With respect to kidney function, we can only speculate about the possible significance of inhibition of NHE3 by adenosine. NHE3 plays a role in net  $\text{Na}^+$  and  $\text{HCO}_3^-$  reabsorption in renal tubules. Short term regulation of NHE3 activity may be

important in changing intraluminal sodium concentrations and altering  $\text{Na}^+$  reabsorption by the kidney, thus favouring reduction of oxygen consumption during ischaemic episodes. Under ischaemic conditions adenosine-dependent inhibition of NHE3 activity may also regulate the rate of bicarbonate reabsorption in the vulnerable outer renal medulla, which is routinely hypertonic. Furthermore, following intrarenal administration of adenosine, inhibition of NHE3 could explain the loss of  $\text{Na}^+$  that is not attributable to changes in renal haemodynamics (Yagil *et al.* 1994). In addition, responsiveness of NHE3 may also explain rapid reduction of salt and volume re-absorption in the proximal tubule that accompanies blood pressure challenge. It is noteworthy in this regard that Zhang and co-workers (Zhang *et al.* 1996) observed that acute pressure natriuresis promoted a removal of apical  $\text{Na}^+-\text{H}^+$  exchangers in the proximal tubule. The validity of the above assumptions, however, remain to be established.

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