J. Membrane Biol. 130, 105–114 (1992)

The Journal of
Membrane Biology

Na^+/H^+-Exchange in A6 Cells: Polarity and Vasopressin Regulation

Valeria Casavola*, Lorenzo Guerra*, Corinna Helmle-Kolb, Stephan J. Reshkin** and Heini Murer
Department of Physiology, University of Zürich, CH-8057 Zürich, Switzerland

Summary. We have analyzed the mechanism of Na^+-dependent pH_i recovery from an acid load in A6 cells (an amphibian distal nephron cell line) by using the intracellular pH indicator 2′7′-bis(2-carboxyethyl)5,6 carboxyfluorescein (BCECF) and single cell microspectrofluorometry. A6 cells were found to express Na^+/H^+-exchange activity only on the basolateral membrane: Na^+/H^+-exchange activity follows simple saturation kinetics with an apparent K_m for Na^+ of approximately 11 mM; it is inhibited in a competitive manner by ethylisopropylamiloride (EIPA). This Na^+/H^+-exchange activity is inhibited by pharmacological activation of protein kinase A (PKA) as well as of protein kinase C (PKC). Addition of arginine vasopressin (AVP) either at low (subnanomolar) or at high (micromolar) concentrations inhibits Na^+/H^+-exchange activity; AVP stimulates IP_3 production at low concentrations, whereas much higher concentrations are required to stimualte cAMP formation. These findings suggest that in A6 cells (i) Na^+/H^+-exchange is located in the basolateral membrane and (ii) PKC activation (heralded by IP_3 turnover) is likely to be the mediator of AVP action at low AVP concentrations.

Key Words Na^+/H^+-exchange · protein kinase A · protein kinase C · vasopressin · distal tubule · tissue culture

Introduction

Na^+/H^+-exchange activity has been found in practically all cell types and is known to serve "housekeeping" functions, including defense of cell pH, participation of control of cell proliferation and in control of cell volume [for review *see:* 13, 15, 18, 42]. In epithelial cell systems, Na^+/H^+-exchange serves additional functions, e.g., in transcellular transport of NaCl and $NaHCO_3$. These specific epithelial functions require polarized expression of Na^+/H^+-exchange activity or at least some "special" control/properties of dually located (apical *vs.* basolateral) exchange activities. For kidney tubular epithelia, for example, apical and/or basolateral location of Na^+/H^+-exchange activity has been described: apical Na^+/H^+-exchange, found mainly in the apical (brush border) membrane of the proximal tubules, serves tubular reabsorption of NaCl and $NaHCO_3$; basolateral Na^+/H^+-exchange, found in some specific parts of the proximal tubule (mainly S_3 of superficial nephrons and entire proximal tubule of juxtameduallary nephrons) as well as in all other nephron segments, may serve mainly "housekeeping" functions [for review *see:* 1, 13, 22, 38].

Tissue culture cells of renal epithelial origin (mostly proximal tubular) have been useful in studying the polarized expression of Na^+/H^+-exchange allowing for a characterization of its kinetic features (e.g., sensitivity to inhibition by amiloride analogues) and for studies of its regulatory properties. Such studies provided evidence for apical location of Na^+/H^+-exchange [*e.g., OK-cells:* 21, 31], basolateral location of Na^+/H^+-exchange [*MDCK-cells:* 43, 50; compare with: 34; *LLC-PK_1-cells:* 30] as well as dual location of Na^+/H^+-exchange (*LLC-PK_1-PKE_{20}-cells:* 8, 9, 10, 19; *MCT-cells:* 32]. With respect to amiloride sensitivity, above studies indicated that apically located Na^+/H^+-exchange is more resistant to inhibition than basolaterally located activity [8, 9, 10, 19; for review *see:* 13]; on the other hand, above studies also indicated, that apical activity is inhibited by activation of protein kinase C whereas basolaterally located Na^+/H^+-exchange activity is stimulated [8, 9, 10, 21, 32]. It must be assumed at present that these different properties of apically and basolaterally located activities are most likely related to different forms of the Na^+/H^+-exchanger molecule; the basolateral form seems to correspond to the "housekeeping" form structurally identified by Pouysségur and colleagues; the apical or brush border membrane Na^+/H^+-exchanger seems to be not yet structurally identified [29, 41, 44; for review *see:* 13, 22, 38].

In the present study we have used A6 cell mono-

* *Present address:* University of Bari, Institute of Physiology, Via Amendola 165/A, I-70126 Bari, Italy.
** *Present address:* National Institutes of Health, Building 10, Room 1A06, Bethesda, Maryland 20892.

layers to study the cellular location of Na^+/H^+-exchange and its control by vasopressin. A6 cells are of amphibian origin and serve as a model for tight distal tubular epithelia; they are sensitive to aldosterone and to vasopressin [14, 20, 25, 35, 36, 39, 40]. This present study is the first to report basolateral location of Na^+/H^+-exchange in this cell line and to show inhibition of this transport activity by vasopressin. We provide evidence that the vasopressin effect may be related to hormone-dependent activation of both, protein kinase A and protein kinase C regulatory pathways.

Materials and Methods

Cell Culture

A6 cells were obtained from B.C. Rossier (Institute of Pharmacology of Lausanne, Switzerland) at serial passage 78. All experiments were carried out on passages 80–100. Cells were grown at 28°C in a humidified atmosphere containing 5% CO_2 in a modified HAM amphibian medium [36] supplemented with 5% fetal bovine serum (Amimed). Cells were subcultured every 7–10 days; an isotonic NaCl solution (240 mOsm; see below) containing 0.1% trypsin and 1 mM EGTA was used for subculturing.

Solutions

Cells were removed from culture and initially placed in a medium (Na medium) resembling the culture medium in its ionic content and osmolarity (240 mOsm), containing (in mM): 110 NaCl, 3 KCl, 1 $CaCl_2$, 1 KH_2PO_4, 0.5 $MgSO_4$, 5 glucose and 10 HEPES buffered with Tris to a final pH = 7.5. A Na^+-free medium was made by replacing Na^+ with tetramethylammonium (TMA medium). In some experiments Na and TMA media were blended together in order to give intermediate Na^+ concentrations. All solutions were without added bicarbonate; to manipulate intracellular pH, cells were exposed to either Na or TMA medium to which NH_4Cl (20 mM) was added.

Intracellular pH Measurements

Intracellular pH (pH_i) was routinely examined in single A6 cells within a confluent monolayer by using a microscope based fluorometric method (see below). For experiments 2.5×10^5 cells/cm^2 were seeded onto collagen-coated Teflon filters (Millicell-CM; Millipore) attached to plastic coverslips having a 1.5 mm hole in the center [filterslips; 30]. In this way, the cell monolayers had access to medium from both sides of the filters. After seeding, cells were returned to 5% CO_2 in 95% air at 28°C for 2 hr. Unattached cells were then removed and filterslips returned to culture. Cells formed a confluent monolayer within 3–4 days and were used for experiments 1–3 days after confluency. For measurements of pH_i, confluent cell monolayers were loaded with BCECF by exposure to 10 μM of the acetoxymethyl ester for 60 min at room temperature in Na medium. This preincubation was carried out in the presence of 25 μM probenecid to prevent dye from leaking from the cells during the loading procedure. Cells on filters (filterslips) were then inserted into a chamber that allowed independent superfusion of the apical and basolateral compartments, as previously detailed [30, 31].

The computerized microfluorometer and the optical components used for the measurements of the fluorescence of BCECF emitted from single cells have been described previously [30]. These measurements were made in response to 7-msec pulses of light from a 75 W Xenon lamp. Sequential excitation of the dye was carried out using two band pass filters: 390 to 440 nm and 475 to 490 nm. The resultant fluorescent emissions (515–565 nm) were corrected for cellular autofluorescence. Calibration of BCECF fluorescence *vs.* a given pH_i was performed with the nigericin-high-K^+ technique, as described previously [31].

Cyclic AMP Determination

Hormone-dependent formations of cyclic AMP (cAMP) were measured essentially as previously described [10]; the procedure is based on a competition protein binding assay [6]. A6 cell monolayers grown on collagen coated filter inserts were treated for 5 min with vasopressin in the presence of 1 mM isobutylmethylxanthine (IBMX).

Inositol (1,4,5)-Triphosphate (IP_3) Measurements

IP_3 measurements in cells grown on filter inserts were performed essentially as described previously [10]; the procedure is based on a competition protein binding assay [5, 33]. Cells on filter inserts were incubated with culture medium containing 20 μM LiCl for 20 min, in order to inhibit dephosphorylation of inositol phosphates. This medium was removed and replaced with culture medium containing 20 μM LiCl and the hormones, as given in the Results section. Cells were incubated for an additional 20 sec, prior to the additional procedures required for IP_3 determinations [10].

Statistics

Significance was determined using a two-tailed t-test for paired or unpaired data. A value of $P < 0.05$ was assumed to indicate a significant difference. All values are presented as means ± SE.

Chemicals

BCECF-AM was purchased from Molecular Probes (Eugene, OR). EIPA (ethylisopropylamiloride) was obtained from Dr. G. Burckhardt (Frankfurt) and was added to solutions from a 20-mM stock solution in dimethylsulfoxide (DMSO). Arginine vasopressin (AVP) was added in the respective salt solution, forskolin in ethanol and TPA in DMSO; these compounds were obtained from Sigma Chemical (St. Louis, MO). Cyclic[^{3}H]AMP ([^{3}H]cAMP) and [^{3}H]inositol (1,4,5)-triphosphate ([^{3}H]InsP_3) were obtained from New England Nuclear (Switzerland). Culture materials were from Amimed (Switzerland) and GIBCO (Switzerland).

Abbreviations

HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid; EIPA: ethylisoproylamiloride; pH_i: intracellular pH; cAMP: adenosine 3′5′-cyclic monophosphate; TPA: phorbol 12-myris-

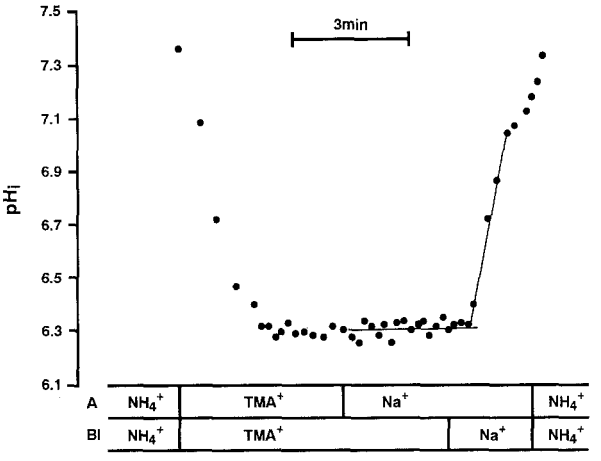

Fig. 1. Na^+ dependence and polarity of pH_i recovery from an acid load in A6 cell monolayers. An acid load was imposed by exposing cells to Na medium containing 20 mM NH_4Cl for an interval of 4–5 min, prior to changing to TMA medium (sodium-free medium). After an additional 5–6 min, the apical (*A*) or basolateral (*Bl*) cell compartment was perfused with Na medium or TMA medium as indicated in the figure. As shown, the addition of basolateral Na medium is sufficient to initiate a pH_i recovery.

tate 13-acetate; EGTA: ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid; Tris: 2-amino-2 hydroxymethylpropane-1,3-diol; BCECF-AM: 2′7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester; IP_3: inositol (1,4,5)-triphosphate; AVP: arginine vasopressin; TMA: tetramethylammonium.

Results

Polarized Recovery of Intracellular pH from an Acid Load

The steady state intracellular pH (pH_i) of A6 cells examined by microspectrofluorometry averaged to 7.3 ± 0.2 (n = 28). This pH_i value was recorded with Na^+-containing solutions (Na medium) as perfusates on both sides. To test for the presence of a Na^+/H^+-exchange activity, we monitored the recovery of pH_i from an acid load. For this purpose the cell interior was acidified by using the NH_4Cl prepulse technique, commonly employed to acidify the cell interior and thereby to activate Na^+/H^+-exchange [4]. Figure 1 illustrates the time course of a typical experiment in which we examined the sidedness of the Na^+-dependent pH_i recovery in single A6 cells. Data presentation starts immediately after the replacement of Na medium containing 20 mM NH_4Cl with TMA medium. After a brief (4 min) exposure to 20 mM NH_4Cl, A6 cells remained acidified in the presence of TMA medium on both sides. The addition of 110 mM Na^+ to only the apical side induced practically no pH_i recovery, while Na^+ added to the serosal side produced a rapid pH_i recovery (pH/min = 0.294 ± 0.019; n = 22), reflecting the activity of a sodium-dependent acid extruding transport system at the basolateral membrane. To further substantiate basolateral location of the Na^+-dependent acid extrusion system we also have made limited experiments on cells grown on the same filter support material, but analyzed in a routine spectrofluorometer employing a specially designed perfusion cuvette [24]. Also in this experimental set up we have only seen basolateral location of Na^+-dependent proton extrusion (*data not shown*).

Effect of Ethylisopropylamiloride (EIPA)

To examine whether the basolateral Na^+-induced pH_i recovery was due to a Na^+/H^+-exchange activity, we analyzed for an effect of EIPA, a specific inhibitor of Na^+/H^+-exchange [e.g., 49]. In these experiments pH_i recovery in response to the addition of 110 mM Na^+ to the basolateral surface of the monolayer was analyzed both in the absence and presence of 5 μM EIPA (Fig. 2). To allow comparison of control and experimental pH_i-recovery rates, cells were verified to show reproducible rates of Na^+-dependent pH_i recoveries after repeated acidifications. The results of Fig. 2 demonstrate that 5 μM EIPA completely inhibited the Na^+-dependent pH_i recovery and suggest that it was entirely due to the operation of Na^+/H^+-exchange. Similar observations were made with the perfusion cuvette system (*data not shown*).

Na^+-Concentration-Dependence of pH_i Recovery and EIPA Interaction

The Na^+-activation kinetics of the basolateral Na^+-dependent pH_i recovery process are illustrated in Fig. 3. As shown, with increasing external Na^+ concentrations, the rate of cytosolic pH_i recovery showed simple saturation kinetics (Fig. 3). The double-reciprocal plot of the data is shown in the inset of Fig. 3. The values fit a straight line giving an apparent K_m for Na^+ of 11.13 ± 1.27 mM and a maximal rate of recovery, V_{max}, of 0.355 ± 0.013 pH/min.

The inhibitory effect of the amiloride analogue EIPA on Na^+-dependent pH_i recovery was further analyzed at 2 μM and at three different Na^+ concentrations (5, 14, and 110 mM). As demonstrated in Fig. 4, the EIPA inhibition was released in a dose-dependent manner as the Na^+ concentration in-

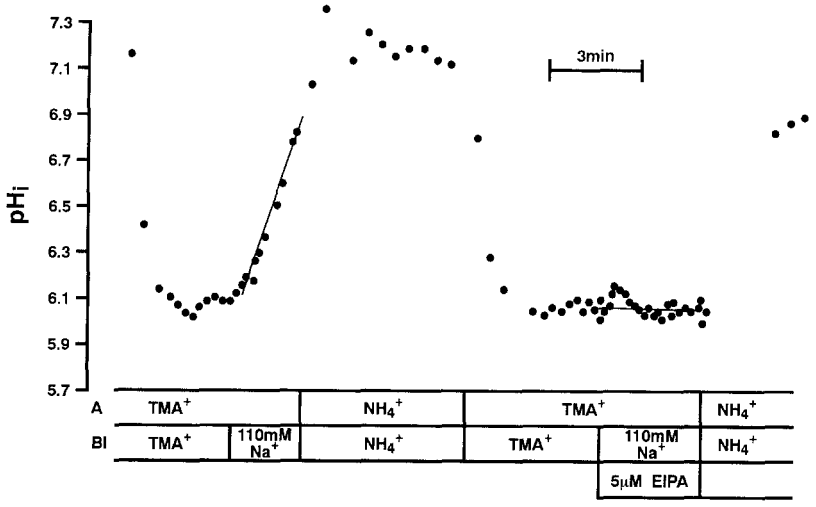

Fig. 2. Effect of 5 μM EIPA on basolateral Na^+-dependent pH_i recovery. Experimental procedures are identical to those described in Fig. 1. The trace is representative of four different experiments. As shown, the presence of 5 μM EIPA prevents completely Na^+-dependent pH_i recovery.

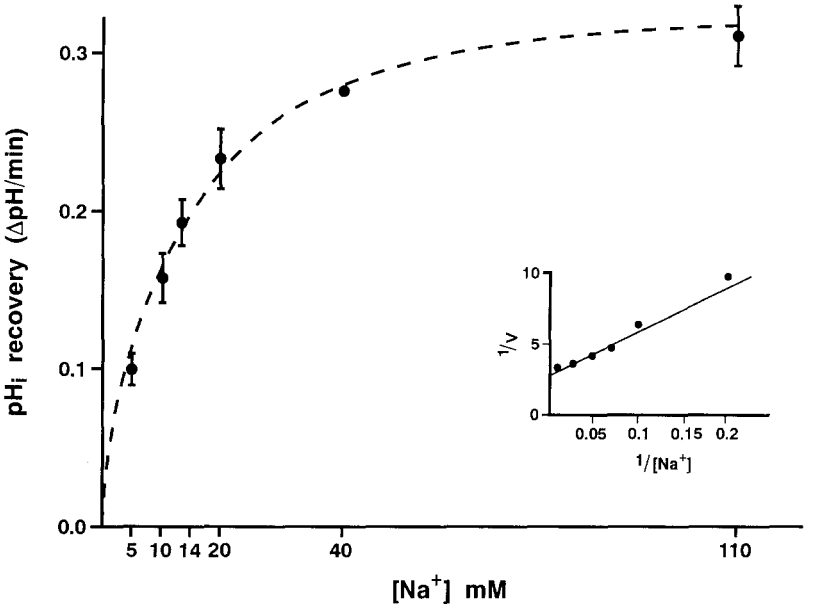

Fig. 3. Na^+-dependency (saturation) of the pH_i recovery. Rates of pH_i recovery from an NH_4-induced acid load (for details *see* Fig. 1) were measured in response to 5 mM (n = 7), 10 mM (n = 5), 14 mM (n = 3), 20 mM (n = 3), 40 mM (n = 1) and 110 mM (n = 14) Na^+ in the basolateral perfusion medium. To examine the effect of a series of different Na^+ concentrations within one monolayer, cells were repeatedly acidified to the same acid intracellular pH value. Na^+ activation of basolateral Na^+/H^+-exchange obeyed Michaelis-Menten kinetics. The Lineweaver-Burk plot of the data is reported in the inset; the line was calculated by linear regression analysis (r = 0.97) and results in an apparent K_m of 11.13 ± 1.27 mM Na^+ and an apparent V_{max} of 0.355 ± 0.013 pH/min.

creased. These data suggest that Na^+ may compete with EIPA for access to the exchanger and are consistent with known properties of the Na^+/H^+-exchange system [for review *see:* 13, 23].

Effect of Pharmacological Activation of Protein Kinase C (PKC) and Protein Kinase A (PKA)

The role of protein kinase C was evaluated using TPA (phorbol 12-myristate 13-acetate), a well-known activator of the protein kinase C regulatory pathway [11]. The basolateral Na^+-dependent pH_i recovery was measured in response to 14 mM Na^+, both in the absence and in the presence of 100 nM TPA. We chose to analyze the pH_i recovery in response to TPA at a sub-maximal Na^+ concentration in order to better observe a variation in the rate of recovery. A 20-min preincubation period with TPA induced an approximately 40% inhibition of the Na^+/H^+-exchange activity (from 0.103 ± 0.019 to 0.058 ± 0.01 pH/min; pH_i recoveries, before and after TPA treatment, respectively, n = 4). In order to exclude that this effect was related to downregulation of PKC, the time of TPA preincubation was reduced to 3 min. (Fig. 5). In this condition the magnitude of inhibition of Na^+/H^+-exchange was approximately the same as observed with the longer (20 min) period of preincubation (the control Na^+-dependent pH_i recovery of 0.079 ± 0.004 decreased to 0.048 ± 0.004 pH/min in the presence of TPA, n = 3).

To evaluate the role of an activation of cAMP-

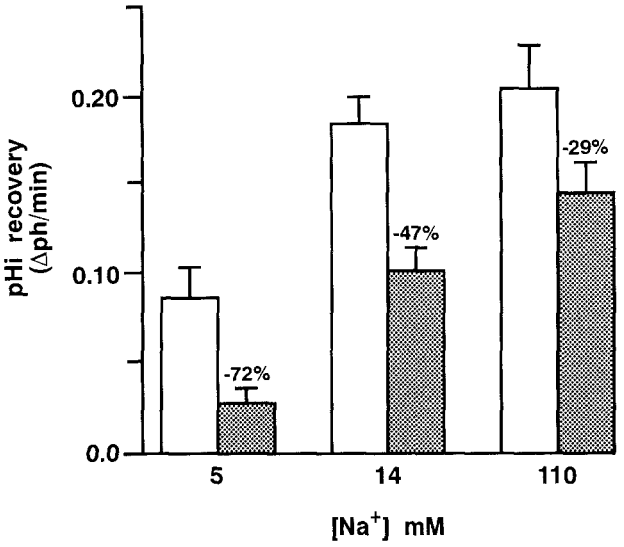

Fig. 4. Inhibition of Na^+/H^+-exchange by 2 μM EIPA. A6 cell monolayers were acidified by the NH_4-prepulse technique to a pH_i ranging from 6.2 to 6.3. Measurements of pH_i recovery of single cells from this acid load were analyzed in response to 5, 14, and 110 mM Na^+, respectively, both in absence and in presence of 2 μM EIPA. Each column represents the mean ± SE of at least three determinations. Note that the percentage of inhibition observed at 110 mM Na^+ in the presence of EIPA (29%) was significantly different ($P < 0.001$) from that observed at 14 mM Na^+ (72%). (□) Control; ▩ 2 μm EIPA.

dependent protein kinase A in the regulation of basolateral Na^+/H^+-exchange activity, we analyzed for an effect of forskolin, an activator of the adenylate cyclase [28]. Figure 6 illustrates the effect of 10 μM forskolin on the ability of the A6 cells to recover from an acid load in the presence of serosal Na^+. We found that 3 min of preincubation with 10 μM forskolin significantly inhibited the serosal pH_i recovery in response to 14 mM Na^+ (0.095 ± 0.003 and 0.072 ± 0.003 pH/min before and after forskolin treatment ($n = 3$, 24% inhibition, $P < 0.001$)). A longer period of forskolin preincubation increased the magnitude of inhibition: the rate of Na^+-dependent pH_i recovery was 0.111 ± 0.016 in the control condition and decreased to 0.076 ± 0.012 pH/min ($n = 4$, 33% inhibition, $P < 0.001$) when 10 μM forskolin was present for 20 min in both compartments before inducing the pH_i recovery.

Effect of Vasopressin on Na^+/H^+-Exchange Activity in A6 Cell Monolayers

A6 cell monolayers, when grown on permeable support, are known to express receptors for vasopressin (AVP) coupled to adenylate cyclase [39]. Thus, it was of interest to analyze for an effect of AVP on Na^+/H^+-exchange activity. The pH_i recovery in response to the basolateral addition of 14 mM Na^+ was analyzed first in the presence of the vehicle of the vasopressin, and then after 10 min of preincubation with 10^{-10} M AVP (Fig. 7). The magnitude of inhibition after 10-min exposure to the hormone was about 40% and approximately the same when AVP preincubation was reduced to 2 min (*data not shown*).

To examine the dose dependency of the sensitivity of A6 cell monolayers to AVP, we analyzed Na^+/H^+-exchange activity in the presence of several AVP concentrations (Table), following the same experimental protocol as described in Fig. 7. Surprisingly, as shown in the Table, the percentage of inhibition induced by vasopressin was almost the same at both the relatively high pharmacological concentrations (10^{-6} to 10^{-7} M) and at more physiological concentrations (10^{-12} M; 10^{-14} M). Similar findings on AVP inhibition of basolateral Na^+/H^+-exchange in A6 cells were also observed in a cuvette perfusion system allowing for population measurements [24; *data not shown*].

cAMP and IP_3 Intracellular Levels in Response to Vasopressin

Since we found that pharmacological activation of either PKC or PKA mimics the AVP effect on the Na^+/H^+-exchange activity (see above), it was of interest to determine the levels of the intracellular messengers cAMP and IP_3 as induced by AVP in order to clarify the transducing mechanism employed by the hormone to inhibit Na^+/H^+-exchange activity in A6 cell monolayers.

In Fig. 8 the levels of the intracellular messengers cAMP (Fig. 8*A*) and IP_3 (Fig. 8*B*) induced by various concentrations of AVP are reported. As shown in this figure, AVP caused an increase both in cellular cAMP and in IP_3 levels in a dose-dependent manner. However, IP_3 production induced by subnamolar concentrations of AVP was considerable (six times with respect to the basal level at 10^{-12} M AVP), whereas cAMP generation was barely detectable at a tenfold higher AVP concentration (10^{-11} M).

Discussion

A6 toad kidney epithelial cells in culture have been used previously as a model system for studies of the regulation of sodium transport in the distal nephron. Although the characteristics of the epithelial sodium transport and its regulation have been widely defined [20, 25, 27, 36, 45], the identity

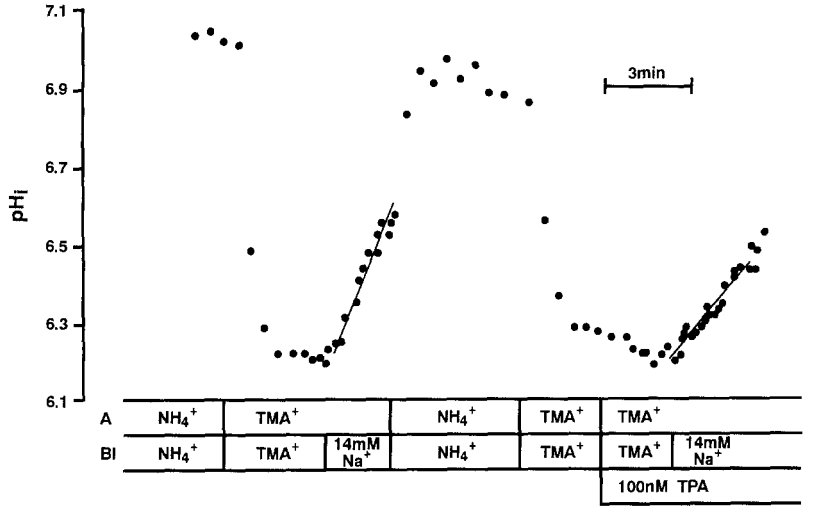

Fig. 5. Effect of 100 nM TPA preincubation on Na^+-dependent pH_i recovery. Paired measurements of initial rate of pH_i increase, induced by addition of 14 mM basolateral Na^+ to acid-loaded cells in the absence or presence of TPA, were performed on cells which were repeatedly acidified to the same acid intracellular pH value. In the experiment shown, acid loaded cells were incubated with TPA from the apical and basolateral side for 3 min before pH_i recovery was examined. Addition of TPA leads to inhibition of Na^+-dependent pH_i recovery.

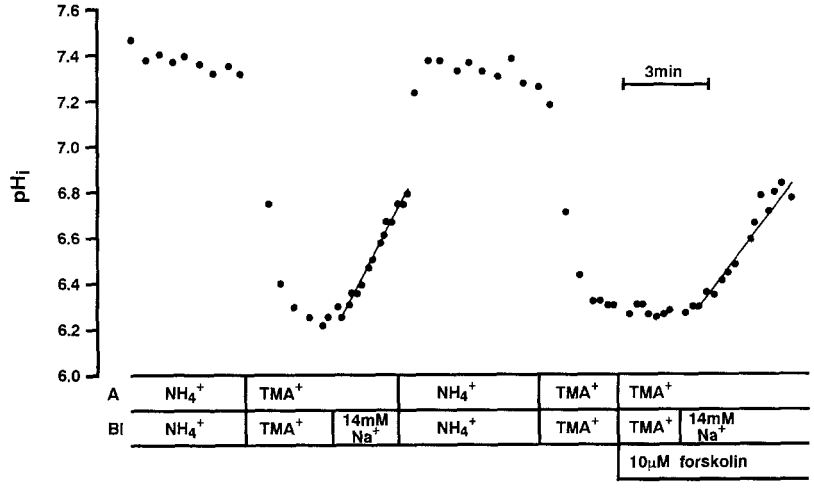

Fig. 6. Effect of 10 μM forskolin on Na^+-dependent pH_i recovery. Na^+-dependent pH_i recovery in the absence and presence of forskolin was assayed under conditions identical to those used for the experiment shown in Fig. 5. Forskolin was added to both sides of the A6 cell monolayer 3 min before the addition of Na^+. Addition of forskolin leads to inhibition of Na^+-dependent pH_i recovery.

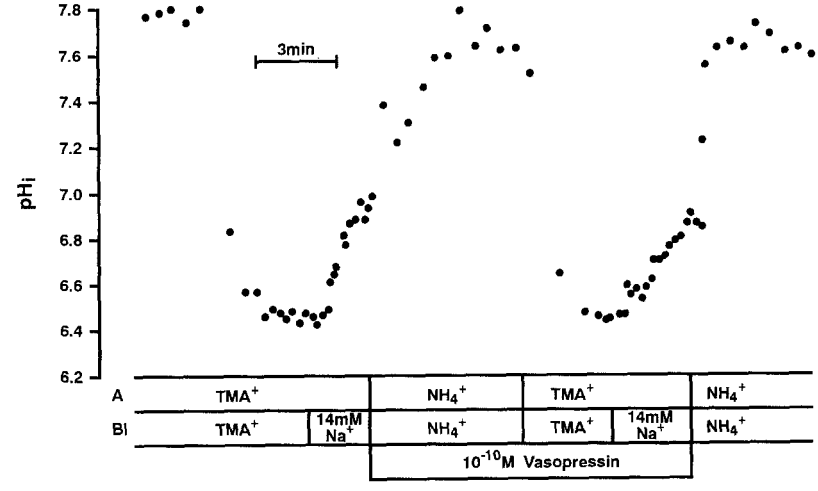

Fig. 7. Effect of 10^{-10} M vasopressin on Na^+/H^+-exchange. Initial rates of pH_i recovery of acid loaded cells under control and vasopressin-stimulated condition were assayed as described in Fig. 5. Cells were exposed for 10 min to vasopressin (e.g., vasopressin was added to the perfusate during an acid load imposed by application and withdrawal of 20 mM NH_4, and was present thereafter). 10^{-10} M vasopressin leads to inhibition of Na^+-dependent pH_i recovery.

Table. Effect of vasopressin on Na^+/H^+ exchange activity in A6 cell monolayers

Hormone inhibition concentration	*n*	Control	Treated	%
		pH/min	pH/min	
$2 \cdot 10^{-6}$ M AVP	(4)	0.137 ± 0.006	0.073 ± 0.013	47 ± 10
$2 \cdot 10^{-7}$ M AVP	(4)	0.094 ± 0.007	0.047 ± 0.005	50 ± 4
$2 \cdot 10^{-9}$ M AVP	(3)	0.120 ± 0.013	0.070 ± 0.012	42 ± 6
$2 \cdot 10^{-10}$ M AVP	(4)	0.127 ± 0.012	0.067 ± 0.009	48 ± 3
$2 \cdot 10^{-12}$ M AVP	(2)	0.093; 0.075	0.030; 0.057	68; 44
$2 \cdot 10^{-14}$ M AVP	(2)	0.077; 0.096	0.028; 0.055	64; 43

Rates of Na^+/H^+-exchange activity in single A6 cells were obtained as described in Fig. 1. Rates of pH_i recovery caused by addition of 14 mM Na^+ either in control conditions or after about 10 min of preincubation with vasopressin (for details of protocol, *see* Fig. 7) are presented. Values are means ± SE. % inhibition is calculated by taking the difference (control − treated) and using the control value as 100%.

and the location of the mechanisms by which intracellular pH is regulated in A6 cells have not been yet examined.

In the first part of this study, A6 cells grown to confluency on a permeable support were examined for polarized distribution and characteristics of the Na^+-dependent proton extrusion mechanism. Recovery of pH_i from an acid load was examined in bicarbonate-free solutions to minimize the likelihood that Na^+-dependent HCO_3^- transport could be responsible for the observed pH_i changes. Microspectrofluorometric studies using BCECF demonstrate that the Na^+-dependent mechanism involved in pH_i recovery from an acid load is confined to the basolateral domain of A6 cells. Furthermore, the EIPA sensitivity strongly suggested that this Na^+-dependent pH_i regulation is due to operation of a basolateral Na^+/H^+-exchanger. The basolateral location of Na^+/H^+-exchange in A6 cells shows similarities to the distal nephron location of Na^+/H^+-exchange: in the cortical collecting tubule both the principal cells and the β-intercalated cells display a basolateral Na^+/H^+-exchanger [12, 51].

In the current study we also characterized some qualitative aspects of Na^+/H^+-exchange inhibition by the potent amiloride analogue ethylisopropylamiloride (EIPA) [49]. We found that in A6 cells the Na^+/H^+-exchanger is highly sensitive to EIPA; the addition of 5 μM EIPA to A6 cell monolayers was sufficient to block completely the activity of the exchanger and the inhibition of Na^+-dependent pH_i recovery by sub-maximal EIPA concentrations (2 μM) was reduced by increasing Na^+ concentrations, suggesting competition of EIPA and Na^+ for binding sites. The high sensitivity of Na^+/H^+-exchange to inhibition by EIPA also suggests that the basolaterally located Na^+/H^+-exchange mechanism falls into the category usually involved in housekeeping functions [for review *see:* 13, 22, 38].

In the second part of the present study, we analyzed for an action of vasopressin on the basolaterally located Na^+/H^+-exchange activity in A6 cells. Our data demonstrate that vasopressin, either at (pharmacologically) high concentrations (10^{-6}, 10^{-7} M) or at (more physiologically) low concentrations (10^{-12} M; 10^{-14} M), is able to inhibit the Na^+/H^+-exchanger. These results differ from those obtained for AVP action on Na^+/H^+-exchange in other preparations [e.g., 3, 16; for review *see:* 13] in which AVP appears to stimulate the Na^+/H^+-exchange activity. It is of interest to note, however, that Ganz et al. [16] in mesangial cells, as well as Berk et al. [3] in cultured vascular smooth muscle cells, found that a transient hormone induced acidification followed by subsequent alkalinization. Moreover, recently, in LLC-PK_1/PKE_{20} cells we found that AVP is able, in the same cell, to stimulate the basolateral and to inhibit the apical Na^+/H^+-exchange activity [10]. The EIPA-sensitivity of the A6 exchanger suggests a close functional homology to the LLC-PK_1/PKE_{20} basolateral Na^+/H^+-exchanger and to the human fibroblast exchanger [for review *see* 13]. It is therefore possible that the difference in AVP response of Na^+/H^+-exchangers may vary with the cell type of context.

Previous studies regarding the effect of phorbol ester in modulating the Na^+/H^+-exchange activity have also yielded "conflicting" results [for review *see:* 13]: For example, activation of protein kinase C resulted in a stimulation of the Na^+/H^+-exchange activity in isolated brush border membranes of renal proximal tubule [e.g., 52]. On the other hand, a phorbol ester induced (protein kinase C mediated) inhibition of apically located Na^+/H^+-exchange was found

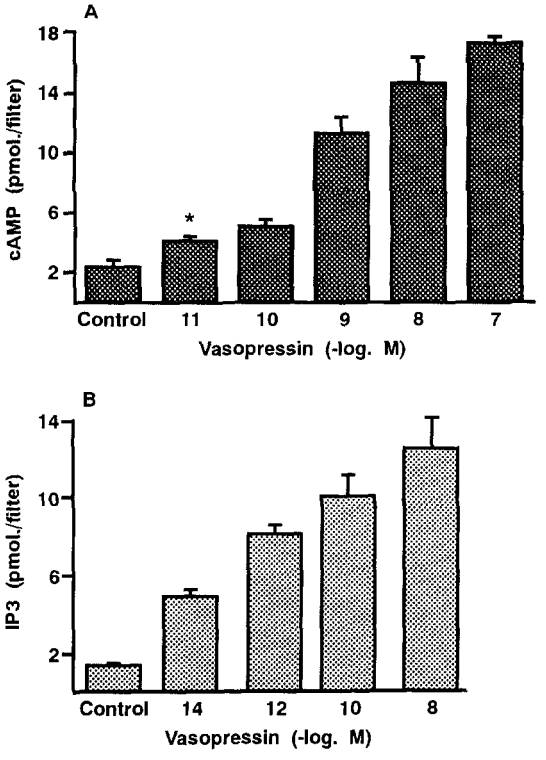

Fig. 8. Effect of various concentrations of vasopressin on the generation of cAMP (*A*) and IP_3 (*B*). For cAMP determinations (*A*) A6 cell monolayers were exposed to varying concentrations of vasopressin for 5 min in the presence of 1 mM IBMX. For the IP_3 determinations (*B*) monolayers were incubated with various concentrations of vasopressin for 20 sec. Levels of internal messengers were determined as described in Materials and Methods. Values are means ± SE for three replicates of a representative experiment. * = NS; all the other conditions resulted in significant effects ($P < 0.05$) as compared to controls.

in some renal cell lines [8, 10, 21, 32]. In the current study we found that pharmacological activation of protein kinase A (forskolin) as well as of protein kinase C (phorbol ester) led to an inhibition of Na^+/H^+-exchange activity up to 40%. We can exclude that the inhibition of Na^+/H^+-exchange activity in A6 cell monolayers was a result of the downregulation of PKC because the same magnitude of inhibition was also observed after a short time of treatment (2 min).

The vasopressin-induced inhibition of the Na^+/H^+-exchange activity in A6 cells cannot be related to a mechanism not directly related to the Na^+/H^+-exchanger, such as to a reduced Na^+-dependent driving force for the serosal antiporter in consequence to an apical Na^+ permeabilization induced by the hormone. We observed the same extent of inhibition of basolateral Na^+-dependent pH_i recovery either when the hormone was present in Na medium for 4 min and then in TMA medium for an additional 4 min before inducing the pH_i recovery (*see* Fig. 7), or when the hormone was added directly to Na^+-free medium only 2 min before the measurement (*data not shown*). Similarly, we can exclude the possibility that the inhibitory effect of AVP on the rate of pH_i recovery is related to an activation of an acid accumulating process, since in the present study we never observed an AVP-effect on pH_i. Therefore, our results strongly suggest that the effect of AVP on Na^+-dependent pH_i recovery is related to a direct action of the hormone on the Na^+/H^+-exchanger mediated by an intracellular messenger cascade system such as an increase in cAMP production and/or by a stimulation of phosphatidylinositol breakdown.

In many AVP-sensitive tissues, including mesangial cells [e.g., 37, 48], the urinary bladder of the toad [26] and the rabbit cortical collecting duct [2, 7, 17], AVP has been demonstrated to stimulate phosphoinositide hydrolysis in addition to the stimulation of the adenylate cyclase. Also in cultured renal epithelia vasopressin stimulation of both adenylate cyclase and phospholipase C has been reported [10, 46, 47]. We also measured the AVP-dependent stimulation of both cAMP and IP_3 production in A6 cells. The dose-dependent AVP-induced stimulation of both cAMP and IP_3 production indicates that in A6 cells the effect of AVP on the Na^+/H^+-exchanger could be mediated by either or both of the two distinct signal transduction mechanisms (adenylate cyclase and/or the phospholipase C message system). However, the observation that AVP stimulated IP_3 production (a marker of protein kinase C activation) at a concentration far lower than that required for cAMP production (Fig. 8), suggests that AVP at physiological (subnanomolar) concentrations could regulate Na^+/H^+-exchange via a mechanism involving protein kinase C. Recent studies regarding the ability of AVP to promote phosphoinositide hydrolysis and cAMP production in rat inner medullary collecting tubules also reported that AVP significantly stimulated IP_3 production at concentrations well below those necessary for stimulation of cAMP production [47].

In summary, these studies provided a first examination of the location and the kinetic characteristics of the Na^+/H^+-exchange activity in A6 cells. We found that A6 cells express a Na^+/H^+-exchange only on the basolateral membrane. This Na^+/H^+-exchange activity is inhibited by the activation of PKA and PKC and by the addition of vasopressin. The physiological significance of the AVP action, the role of PKC and PKA, as well as the possible involvement of calcium in regulating the Na^+/H^+-exchange activity require further elucidation.

This work was supported by the Swiss National Science Foundation (Grant No. 32-30785.91), the Stiftung für wissenschaftliche Forschung an der Universität Zürich, the Hartmann- Müller Stiftung, the Sandoz-Stiftung, the Roche Research Foundation, and the Geigy Jubiläumsstiftung. Prof. Dr. V. Casavola and Dr. R. Guerra were supported by a research grant, No. 91.02470.CT14 of the Consiglio Nazionale della Ricerche (C.N.R.) We are grateful to Prof. Dr. B.C. Rossier of the Institute of Pharmacology of Lausanne (Switzerland) for the gift of the A6 cells, to H.P. Gaeggeler for the supply of the necessary culture media and to Jutka Forgo for her excellent help in the day-to-day culturing of the A6 cells. The secretarial assistance of D. Rossi is gratefully acknowledged.

References

1. Alpern, R.J. 1990. Cell mechanisms of proximal tubular acidification. *Physiol. Rev.* **70:**79–114
2. Ando, Y., Breyer, M.D., Jacobson, H.R. 1989. Dose-dependent heterogenous actions of vasopressin in rabbit cortical collecting ducts. *Am. J. Physiol.* **256:**F556–F562
3. Berk, B.C., Brock, T.A., Gimbrone, M.A., Jr., Alexander, R.W. 1987. Early agonist-mediated ionic events in cultured vascular smooth muscle cells. *J. Biol. Chem.* **262:**5065–5072
4. Boron, W.F., DeWeer, P. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3 and metabolic inhibitors. *J. Gen. Physiol.* **67:**91–112
5. Bredt, D.S., Mourey, R.J., Snyder, S.H. 1989. A simple, sensitive and specific radioreceptor assay for inositol 1,4,5-trisphosphate in biological tissues. *Biochim. Biophys. Res. Commun.* **159:**976–982
6. Brown, B.L., Ekins, R.P., Albano, J.D.M. 1972. Saturation assay for cAMP using endogenous binding protein. *Adv. in Cyclic Nucleotide Res.* **2:**25–40
7. Burnatowska-Hledin, M.A., Spielman, W.S. 1989. Vasopressin V1 receptors on the principal cells of the rabbit cortical collecting tubule. *J. Clin. Invest.* **83:**84–89
8. Casavola, V., Helmle-Kolb, C., Murer, H. 1989. Separate regulatory control of apical and basolateral Na^+/H^+ exchange in renal epithelial cells. *Biochem. Biophys. Res. Commun.* **165:**833–837
9. Casavola, V., Helmle-Kolb, C., Montrose, M.H., Murer, H. 1991. Polarized expression of Na^+/H^+-exchange activities in clonal LLC-PK_1-cells ($Clone_4$ and PKE_{20}): I. Basic characterization. *Pfluegers Arch.* **418:**276–283
10. Casavola, V., Reshkin, S.J., Murer, H., Helmle-Kolb, C. 1992. Polarized expression of Na^+/H^+ exchange activity in LLC-PK_1/PKE_{20} cells: II. Hormonal regulation. *Pfluegers Arch. (in press)*
11. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y. 1982. Direct activation of calcium activated, phospholipid-dependent protein kinase by tumor-promoting phorbol ester. *J. Biol. Chem.* **257:**7847–7851
12. Chaillet, J.R., Lopes, A.G., Boron, W.F. 1985. Basolateral Na-H exchange in the rabbit cortical collecting tubule. *J. Gen. Physiol.* **86:**795–812
13. Clark, J.D., Limbird, L.E. 1991. Na^+/H^+-exchanger subtypes: a predictive review. *Am. J. Physiol.* **261:**C945–C953
14. Fidelman, M.L., Watlington, C.O. 1987. Effect of aldosterone and insulin on mannitol, Na^+, and Cl^- fluxes in cultured epithelia of renal origin (A6): Evidence for increased permeability in the paracellular pathway. *Biochim. Biophys. Acta* **931:**205–214
15. Frelin, C. Vigne, P., Ladoux, A., Lazdunski, M. 1988. The regulation of the intracellular pH in cells from vertebrates. *Eur. J. Biochem.* **174:**3–14
16. Ganz, M.B., Boyarsky, G., Boron, W.F., Sterzel, R.B. 1988. Effect of angiotensin II and vasopressin on intracellular pH of glomerular mesangial cells. *Am. J. Physiol.* **254:**F787–F794
17. Garg, L.C., Kapturczak, E. 1990. Stimulation of phosphoinositide hydrolysis in renal medulla by vasopressin. *Endocrinology* **127:**1022–1027
18. Grinstein, S., Rothstein, A. 1986. Mechanism of regulation of the Na^+/H^+ exchanger. *J. Membrane Biol.* **90:**1–12
19. Haggerty, I.G., Agarwal, N., Reilly, R.F., Adelberg, E.A., Slayman, C.W. 1988. Pharmacologically different Na/H antiporters on the apical and basolateral surfaces of cultured kidney cells (LLC-PK_1). *Proc. Natl. Acad. Sci. USA* **85:**6797–6801
20. Handler, J.S., Steele, R.E., Sahib, M.K., Wade, J.B., Preston, A.S., Lawson, N.L., Johnson, J.P. 1979. Toad urinary bladder epithelial cells in culture: Maintenance of epithelial structure, sodium transport and response to hormone. *Proc. Natl. Acad. Sci. USA* **76:**4151–4155
21. Helmle-Kolb, C., Montrose, M.H., Murer, H. 1990. Parathyroid hormone regulation of Na^+/H^+-exchange in opossum kidney cells: Polarity and mechanisms. *Pfluegers Arch.* **416:**615–623
22. Igarashi, P., Reilly, R.F., Hildebrandt, F., Belmesderfer, D., Rebauças, N.A., Slayman, C.A., Aronson, P.S. 1991. Molecular biology of renal Na^+/H^+ exchangers. *Kidney Int.* **40:**S84–S89
23. Kinsella, J.L., Aronson, P.S. 1980. Properties of the Na^+/H^+ exchanges in renal microvillus membrane vesicles. *Am. J. Physiol.* **238:**F461–F469
24. Krayer-Pawlowska, D., Helmle-Kolb, C., Montrose, M.H., Krapf, R., Murer, H. 1991. Studies on the kinetics of Na^+/H^+ exchange in OK cells: Introduction of a new device for the analysis of polarized transport in cultured epithelia. *J. Membrane Biol.* **120:**173–183
25. Lang, M.A., Preston, A.S., Handler, J.S., Forrest, J.F., Jr. 1985. Adenosine stimulates sodium transport in A6 epithelia in culture. *Am. J. Physiol.* **249:**C330–C336
26. Levine, S.D., Goldin, H., Schlondorff, D. 1989. Vasopressin stimulates phospholipase C activity in toad bladder epithelia membranes. *Clin. Res.* **37:**582*A (Abstr.)*
27. Ling, B.N., Eaton, D.C. 1989. Effect of luminal Na^+ on single Na^+ channels in A6 cells, a regulatory role for protein kinase C. *Am. J. Physiol.* **256:**F1094–F1103
28. Litosh, J., Hudson, T., Mills, J., Li, S.Y., Fain, J.F. 1982. Forskolin as an activator of cyclic AMP accumulation and lipolysis in rat adipocytes. *Mol. Pharm.* **22:**109–115
29. Ming, Tse C., Ma, A.I., Yang, V.W., Watson, A.J.M., Levine, S., Montrose, M.H., Potter, J., Sardet, C., Pouysségur, J., Donowitz, M. 1991. Molecular cloning and expression of a cDNA encoding the rabbit villus cell basolateral Na^+/H^+ exchanger. *EMBO J.* **10:**1957–1967
30. Montrose, M.H., Friedrich, T., Murer, H. 1987. Measurements of intracellular pH in single LLC-PK_1 cells: Recovery from an acid load via basolateral Na^+/H^+ exchange. *J. Membrane Biol.* **97:**63–78
31. Montrose, M.H., Murer, H. 1990. Polarity and kinetics of Na^+/H^+ exchange in cultured opossum kidney cells. *Am. J. Physiol.* **259:**C121–C133
32. Mrkic, B., Forgo, J., Murer, H., Helmle-Kolb, C. 1992. Apical and basolateral Na/H-exchange in cultured murine proxi-

mal tubule cells (MCT): Effect of parathyroid hormone (PTH). *J. Membrane Biol.* **130:**203–215
33. Nibbering, P.H., Zomerdijk, T.P.L., Van Haastert, P.J.M., Van Furth, R. 1990. A competition binding assay for determination of the inositol (1,4,5)-triphosphate content of human leucocytes. *Biochem. Biophys. Res. Commun.* **170:**755–762
34. Oberleithner, H., Vogel, U., Kersting, U., Steigner, W. 1990. Madine-Darby canine kidney cells: II. Aldosterone stimulates Na^+/H^+ and Cl^-/HCO_3 exchange. *Pfluegers Arch.* **416:**533–539
35. Paccolat, M.R., Geering, K., Gaeggeler, H.P., Rossier, B.C. 1987. Aldosterone regulation of Na^+/K^+ ATPase in A6 cells: Role of growth conditions. *Am. J. Physiol.* **252:**C468–C476
36. Perkins, F.M., Handler, J.S. 1981. Transport properties of toad kidney epithelia in culture. *Am. J. Physiol.* **241:**C154–C159
37. Pfeilschifter, J. 1989. Cellular signalling in the kidney: The role of inositol lipids. *Renal Physiol. Biochem.* **12:**1–31
38. Preisig, P.A., Alpern, R.J. 1991. Basolateral membrane H/HCO_3 transport in renal tubules. *Kidney Int.* **39:**1077–1086
39. Preston, A.S., Muller, J., Handler, J.S. 1988. Dexamethasone accelerates differentiation of A6 epithelia and increases response to vasopressin. *Am. J. Physiol.* **255:**C661–C666
40. Rafferty, K.A. 1969. Mass culture of amphibian cells: Methods and observations concerning stability of cell type. *In:* Biology Of Amphibian Tumors. M. Mizell, editor. pp. 52–81. Springer-Verlag, New York
41. Reilly, R.F., Hildebrandt, F., Biemesderfer, D., Sardet, C., Pouysségur, J., Aronson, P.S., Slayman, C.W., Igarashi, P. 1992. cDNA cloning and immunolocalization of a Na^+/H^+-exchanger in LLC-PK_1 renal epithelial cells. *Am. J. Physiol.* **261:**F1088–F1094
42. Roos, A., Boron, W.F. 1981. Intracellular pH. *Physiol. Rev.* **61:**296–434
43. Rosenberg, S.O., Berkowitz, P.A., Li, L., Schuster, V.L. 1991. Imaging of filter-grown epithelial cells: MDCK Na^+-H^+ exchanger is basolateral. *Am. J. Physiol.* **260:**C868–C878
44. Sardet, C., Franchi, A., Pouysségur, J. 1989. Molecular cloning, primary structure and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell* **56:**271–280
45. Sariban Sohraby, S., Burg, M.B., Turner, R.J. 1983. Apical sodium uptake in the toad kidney epithelial cell line A6. *Am. J. Physiol.* **245:**C167–C171
46. Tang, M.J., Weinberg, J.M. 1986. Vasopressin-induced increases of cytosolic calcium in LLC-PK1 cells. *Am. J. Physiol.* **251:**F1090–F1095
47. Teitelbaum, I., Strasheim, A. 1990. AVP stimulates adenylcyclase and phospholipase C in reciprocal fashion in cultured RIMCT cells. *Am. J. Physiol.* **259:**C693–C696
48. Troyer, D.A., Kreisberg, J.I., Schwertz, D.W., Venkatachalam, M.A. 1985. Effect of vasopressin on phosphoinositides and prostaglandin production in cultured mesangial cells. *Am. J. Physiol.* **249:**F139–F147
49. Vigne, P., Frelin, C., Cragoe, E.J., Lazdunski, M. 1989. Ethylisopropylamiloride: A new and highly potent derivative of amiloride for the inhibition of the Na-H exchange system in various cell types. *Biochem. Biophys. Res. Commun.* **116:**86–90
50. Vilella, S., Guerra, L., Helmle-Kolb, C., Murer H. 1992. Characterization of basolateral Na/H-exchange (Na/H-1) in MDCK-cells. *Pfluegers Arch. (in press)*
51. Weiner, D.I., Hamm, L.L. 1990. Regulation of intracellular pH in the rabbit cortical collecting tubule. *J. Clin. Invest.* **85:**274–281
52. Weinman, E.J., Shenolikar, S. 1986. Protein kinase C activates the renal apical membrane Na^+/H^+-exchanger. *J. Membrane Biol.* **93:**133–139

Received 11 March 1992; revised 27 May 1992