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Vasopressin-Dependent Control of Basolateral Na/H-Exchange in Highly Differentiated A6-Cell Monolayers

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Abstract. We have used a well-differentiated A6-cell preparation (A6-C1) to study cellular location and vasopressin control of Na/H-exchange activity. After cell acidification, cell pH_i (measured by BCECF-fluorescence) only recovered by the addition of Na medium to the basolateral cell surface; this pH_i recovery was inhibited by dimethylamiloride (2 μM) consistent with basolateral location of Na/H-exchange activity. Addition of vasopressin produced stimulation of Na/H-exchange activity and increased the affinity of the exchanger for Na^+. Stimulation of Na/H exchange was mimicked by pharmacological activation of protein kinase A (forskolin, 8-Br-cAMP) and not by pharmacological activation of protein kinase C (TPA). It is concluded that basolaterally located Na/H-exchange in A6-C1 cells is activated by vasopressin.

Key words: Na^+/H^+-exchange — Protein kinases — cAMP — Vasopressin — Distal tubule — Tissue culture

Introduction

In renal tubular epithelial cells Na/H-exchange has a dual location and a dual function: (i) Apical Na/H-exchange activity is involved in NaCl- and $NaHCO_3$-reabsorption; (ii) basolaterally located Na/H-exchange activity fulfills mainly "housekeeping" functions [*for review see:* 1, 6, 9, 20]. In addition, intracellular pH was shown to modulate apical Na permeability [7, 18] and basolaterally located Na/H-exchange in "tight" epithelia was suggested to participate in the mediation of hormonal effects, e.g., in the control of Na reabsorption by aldosterone [16].

Studies on cultured renal epithelia have been used to study cellular mechanisms participating in the regulation of Na/H-exchange activities. In particular, cell lines with proximal tubular properties have been used frequently to study hormonal regulation of apically located as well as basolaterally located Na/H-exchange [5, 8, 14, 15]. Studies on MDCK cells, a cell line related to more distal nephron segments [22, 27], have shown aldosterone-dependent activation of Na/H-exchange [16, 28].

A6 cells serve as a model for tight distal tubular epithelia; they are derived from the kidney of *Xenopus laevis* and are sensitive to aldosterone and vasopressin [11, 12, 13, 17, 19, 21, 23, 25, 26; *for review see:* 24]. To optimize cell differentiation and hormonal responsiveness, A6 cells not only require growth on permeant porous supports but also pretreatment with defined serum and/or hormone additions [11, 12, 17, 21, 25, 26]. In a recent study [25], F. Verrey et al. have documented that in clonally selected A6-cell monolayers (A6–C1 cells) aldosterone pretreatment leads to the development of highly differentiated monolayers with high sensitivity to vasopressin (short circuit current, cAMP generation).

In a previous study, we have used A6 cells and have found an exclusive basolateral location of Na/H-exchange and inhibition of this transport activity by vasopressin; however, we did not verify whether

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the selected growth conditions have been adequate to maintain a "physiological" response, i.e., a vasopressin-dependent increase in short circuit current [4]. In the present study, we have used A6-C1 cell monolayers to investigate the polarized expression and vasopressin control of Na/H-exchange. We have used culture conditions in which distal tubular epithelial properties, such as high rates of amiloride-inhibitable short circuit currents and high sensitivity to vasopressin [25] were expressed and could be verified on the same preparation as used subsequently for measurements of Na/H-exchange activity. In agreement with our previous results [4], we found an exclusive basolateral location of Na/H-exchange; in contrast to the observation made with "unconditioned" A6-cell monolayers [4], basolateral Na/H-exchange in the A6-C1 cell preparations was activated by vasopressin, most likely mediated by activation of protein kinase A.

Materials and Methods

Cell Culture

An A6-cell clone (A6-C1) was obtained by ring-cloning of A6-2F3 cells [25, 26]; it was selected on the basis of its high electrical resistance and its responsiveness to aldosterone and antidiuretic hormone [25; F. Verrey et al. *to be published*]. A6-C1 cells were cultured on plastic dishes in 0.8 × concentrated DMEM (GIBCO, Basel, Switzerland) containing 25 mM $NaHCO_3$ and supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Allschwil, Switzerland); 1% of a penicillin-streptomycin mix (GIBCO) was also added to the culture medium and cultures were maintained at 28°C in a 5% CO_2/95% air atmosphere. Cells were subcultured weekly by incubation in Ca^{2+}/Mg^{2+}-free salt solution containing 0.25% (w/v) trypsin and 1 mM EGTA; cells were usually split at 1/10 or 1/20 ratios and culture medium exchanged every 3 days. For culture on permeant filter supports, polycarbonate filters (Transwell, 0.4 μM pore size, 4.7 cm^2, Costar, Cambridge, MA) were coated with a thin layer of collagen (type 1; Vitrogen 100, Collagen, Palo Alto, CA) treated with glutaraldehyde; seeding density was high (cells from a 10 cm^2 confluent monolayer for a 4.7 cm^2 filter insert). Monolayers were kept on filters for at least 10 days under these conditions and growth medium was replaced twice during this period. Approximately 3–5 days prior to pH_i measurements, growth medium was then replaced by serum-free bicarbonate-free 0.8 × DMEM buffered with 20 mM HEPES to a pH 7.4 and containing 10^{-6} M aldosterone and 1% of a penicillin-streptomycin mix; during this time period cells were kept at 28°C in a CO_2-free atmosphere. This treatment with aldosterone was required for potentiating the cellular response to vasopressin, with respect to cAMP generation and increase in short circuit currents, which were magnified about 15 and 30 times by the aldosterone pretreatment, respectively [25; F. Verrey et al., *to be published*]. Only cell monolayers treated with aldosterone have been used in this study.

Measurement of Transepithelial Short Circuit Current

Measurements of transepithelial potential difference (mV) and short circuit current (μA/cm^2) were performed in a modified Ussing chamber according to published methods [17]. Transepithelial resistance (Ω · cm^2) was calculated according to Ohm's law. For the present study the electrical parameters were measured in the Na medium used for pH_i measurements (*see below*) and at room temperature.

Fluorescence (pH_i) Measurements

Intracellular pH (pH_i) was measured using the fluorescent dye BCECF. The polycarbonate filters containing the confluent monolayers were removed from the plastic insert and cut into three pieces. Pieces were kept in Na medium (*see below*, maximally 3 hr) and exposed for 60 min to 16 μM BCECF-AM (in Na medium containing 50 μM probenecid). Subsequently, the filter piece was inserted into a perfusion cuvette between a plastic gasket and a perforated plastic coverslip; the perfusion cuvette is especially designed for separate superfusion of apical and basolateral cell surface [10]. Fluorescence was recorded in a Schimadzu RF 5000 spectrofluorometer using 530 nm (bandwidth 15 nm) as emission wavelength and 500 nm (pH sensitive) and 455 nm (pH insensitive) as excitation wavelengths (bandwidth in both cases 5 nm). pH_i was calculated from the ratios from fluorescence intensities at the two above-mentioned excitation wavelengths by using a standard calibration procedure based on the use of nigericin in high potassium media buffered at different pH values [e.g.: 10, 14].

Composition of Perfusion Solutions

All pH_i measurements were performed at room temperature in HEPES-buffered, bicarbonate-free media. Na medium contained: 110 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 5 mM D-glucose, 0.1% BSA, 5 μM probenecid and 10 mM HEPES [pH adjusted to 7.5 by titration with tris-(hydroxymethyl) aminomethane]. For cellular acidification we have used the NH_4^+/NH_3-loading removal protocol [3]; the NH_4Cl-solution was identical to Na medium and contained in addition 20 mM NH_4Cl. A sodium-free (TMA medium) was obtained by complete replacement of sodium with tetramethylammonium. To obtain solutions of different sodium-concentrations, Na medium and TMA medium were mixed appropriately. The high potassium media used for pH_i calibration contained (mM) 8 NaCl, 105 KCl, 1 $CaCl_2$, 0.5 $MgSO_4$, 1 KH_2PO_4, 5 D-glucose, and 10 HEPES adjusted to different pH values.

Data Evaluation (Statistics)

Because of the large interexperimental variability in transport activity from one cell population to another, and from one passage to another, a repetitive NH_4^+–NH_3 prepulse protocol was used to quantify the influence of effectors on rate of Na/H-exchange activity as follows: pH_i recovery rates were first observed in the absence of effector, and then again in the same cell population at identical acid pH_i in the presence of effector. Adequate controls were performed to ensure that measured pH_i recovery rates after

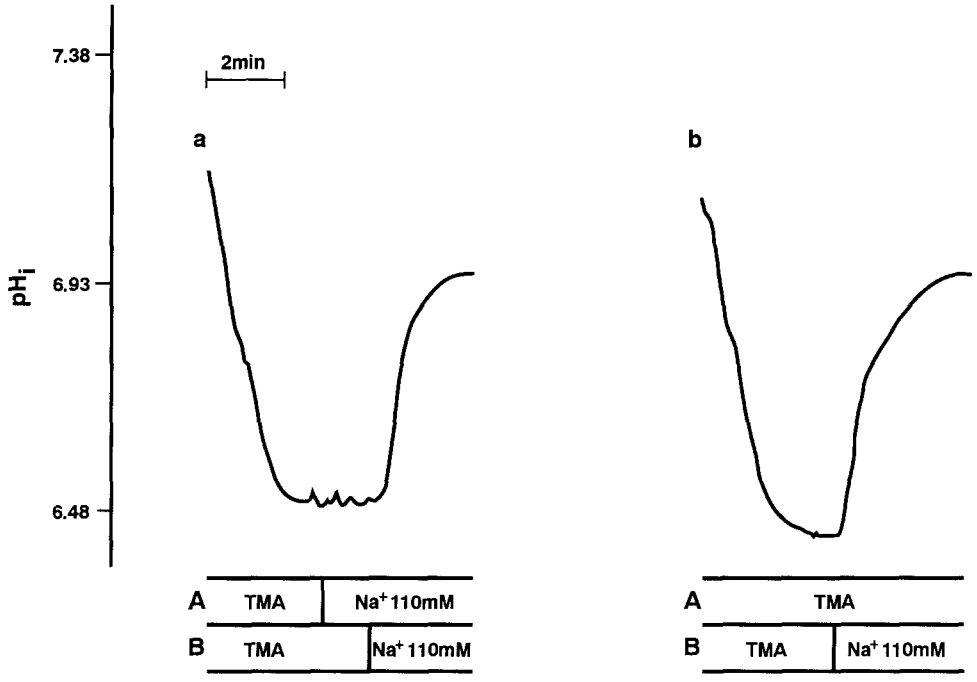

Fig. 1. Na-dependent pH_i recovery in A6-C1 cell monolayers. Cells were acid-loaded by superfusion on both sides with Na medium containing 20 mM NH_4Cl; exposure to this medium was for 6–7 min. NH_4Cl-containing solution was replaced by a TMA solution (beginning of data collection) and the apical and basolateral cell surfaces exposed to different solutions as indicated in the figure; Na concentration was 110 mM. The bars below the traces highlight the perfusion conditions: *A* defines the apical perfusate and *B* the basolateral perfusate. Panels ***a*** and ***b*** represent consecutive cycles of acidification and pH_i recovery performed on the same monolayer.

several NH_4^+/NH_3 prepulses were highly reproducible (*see also* Fig. 1). Data were analyzed using a two-tailed *t*-test for paired or unpaired data. A value of $P < 0.05$ was accepted for statistical significance. Results are expressed as means ± SE. Traces given in figures are representative of three experiments with qualitatively identical observations.

Abbreviations

HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid; EIPA, ethylisopropylamiloride; DMA, dimethylamiloride; pH_i, intracellular pH; cAMP, adenosine 3′5′-cyclic monophosphate; EGTA, ethylene glycol-bis (β-amino-ethylether)-N, N, N′, N′-tetraacetic acid; BCECF-AM, 2′7′-bis (2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester; AVP, arginine vasopressin; TMA, tetramethylammonium; 8-Br-cAMP, 8-bromo-adenosine 3′5′ cyclic monophosphate; TPA, phorbol 12-myristate 13-acetate.

Results and Discussion

Cellular Location and Properties of Na/H-Exchange

As indicated in Fig. 1 (trace *a*), after replacing the NH_4Cl medium by TMA medium (data collection started immediately after this solution change) cell pH_i dropped and did not recover in the absence of sodium. Also, the readdition of Na medium to the apical medium did not lead to a pH_i recovery. A recovery from the acid load (delay of a few seconds due to perfusion system) was observed during superfusion of the basolateral cell surface with Na medium. Figure 1 (trace *b*) shows a similar experiment performed after about 15 min on the same monolayer. It indicates that multiple recovery processes could be recorded on the same monolayer without an apparent loss in transport activity. Under the experimental conditions given in Fig. 1 (110 mM Na) pH_i recovery was fast and reached within 3–5 min a steady pH_i value of 7.0 ± 0.1 (n = 20), reflecting most likely resting pH_i under these perfusion conditions.

To verify that basolateral Na-dependent pH_i recovery is due to Na/H-exchange activity, we have studied the effect of an amiloride analogue (dimethylamiloride, Research Biochemicals, MA) on transport rate. As shown in Fig. 2, the initial rate of Na-dependent pH_i recovery at 5.5 mM basolateral Na (trace *a*) was completely prevented by 2 μM DMA (trace *b*); removal of DMA led to restauration of transport activity (trace *c*). These results are consistent with basolateral location of Na/H-exchange activity in A6-C1 cell monolayers. The present find-

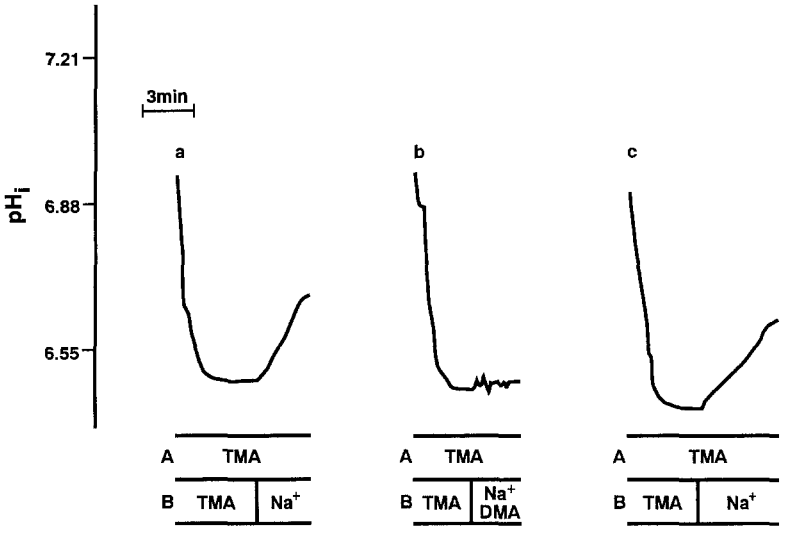

Fig. 2. Inhibition of basolaterally located Na-dependent pH_i recovery by dimethylamiloride (DMA). The experiment was performed in a manner identical to that given in Fig. 1. Na concentration was 5.5 mM and DMA concentration 2 μM. The data show complete inhibition of basolateral Na-dependent pH_i recovery by DMA (compare *a* and *b*) and (partial) recovery from this inhibition (compare *b* and *c*).

Table 1. Fractional change of transepithelial short circuit current due to the addition of either vasopressin, forskolin or 8-Br-cAMP

Hormone/agonists	Short circuit current (test/control)
Arginine-vasopressin, 2.5×10^{-7} M	3.82 ± 0.9*
Forskolin, 10^{-5} M	2.55 ± 0.45*
8-Br-cAMP, 10^{-3} M	1.84 ± 0.10*

Data (means ± SE; $n = 4$) are expressed as fractional change (test/control); in controls (aldosterone-treated A6-C1 monolayers) short circuit current was between 40 to 60 μA/filter [*see also:* 25]. Hormone/agonists were added at the indicated concentrations and alterations in short circuit current recorded after a time period of 10 to 15 min.
* $P < 0.05$, compared to control value.

ings on membrane localization of Na/H-exchange activity are in agreement with recent observations made at the single cell level of A6-2F3 cell monolayers which had not been pretreated with aldosterone [4]. Basolateral location of Na/H-exchange is also in agreement with other reports on distal tubular epithelia [e.g., 22, 27, 28; *for review see:* 6, 20].

Effect of Arginine-Vasopressin on Na/H-Exchange

Under the conditions used throughout this study, the addition of arginine-vasopressin (or vasotocin) caused a "physiological" response, i.e., an increase in cellular cAMP content [*see below;* 25] and a 3- to 4-fold increase (at 2.5×10^{-7} M vasopressin) in short circuit current [*see* Table 1; 25].

In the experiment shown in Fig. 3, a preincubation (≈10 min) of monolayers with 2.5×10^{-7} M arginine-vasopressin led to a stimulation of basolaterally located Na/H-exchange. When measured at an intermediate Na concentration (22 mM); stimulation was about 25% (from 0.22 ± 0.01 to 0.28 ± 0.02 pH/min; $n = 5$; $P < 0.005$). Removal of the agonist resulted in a lower Na/H-exchange rate, comparable to that observed for the initial control value (Fig. 3; compare traces *b* and *c*; reversibility). As Na/H-exchange activity was initiated (Na addition) always at a similar pH_i (maximally accepted difference: 0.05 pH units), a change in dpH/dt following application of an agonist is expected to be most likely a consequence of an agonist-induced alteration of the transport process itself and not a consequence of different cellular acid loads (allosteric control of Na/H-exchange) or of pH-dependent alterations in cellular buffering capacity. Furthermore, that DMA treatment did block pH_i recovery, also in the presence of AVP (*data not shown*), demonstrates that the AVP-induced increase in the rate of pH_i recovery was not due to the activation of a pH regulatory process other than Na/H-exchange.

To characterize further the effect of arginine-vasopressin on Na/H-exchange, transport rates at 5.5 mM Na were examined at a low (2×10^{-10} M) and a high (2.5×10^{-7} M) hormone concentration; the rationale for using a low Na concentration was that, for technical reasons, small alterations in Na/H-exchange activities can be detected more easily at low pH_i recovery rates, and that the relative changes induced by the hormonal treatment were higher at low Na concentration (*see below*). As shown in Table 2, 2×10^{-10} M arginine-vasopressin increased Na/H-exchange activity by 12% and 2.5×10^{-7} M by 44%.

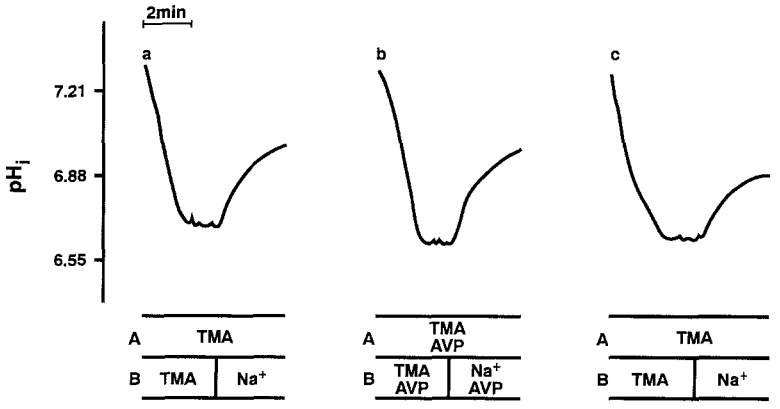

Fig. 3. Stimulation of basolaterally located Na/H-exchange by arginine-vasopressin. The experiments were performed in a manner analogous to that illustrated in Fig. 1. Arginine-vasopressin at a concentration of 2.5×10^{-7} M was already included in the NH_4Cl and TMA solutions and present throughout the recording of pH_i as indicated in the figure; cells were exposed to the hormone for about 10 min prior to initiation of Na-dependent pH_i recovery with 22 mM Na. A comparison between *a* and *b* illustrates hormone-dependent stimulation of Na/H-exchange; comparison between *b* and *c* shows recovery from the hormone effect.

Table 2. Arginine-vasopressin-dependent stimulation of Na/H-exchange, influence of hormone concentration

Arginine-Vasopressin (M)	*n*	Control (Δ pH/min)	Treated (Δ pH/min)	% Stimulation
2×10^{-10}	4	0.067 ± 0.006	0.075 ± 0.006	12.42 ± 0.80*
2.5×10^{-7}	5	0.094 ± 0.007	0.135 ± 0.009	44.19 ± 2.65*

Aldosterone-treated A6-C1 monolayers were acidified and Na-dependent pH_i recovery was analyzed at 5.5 mM sodium in the basolateral perfusate. Arginine-vasopressin was added at two different concentrations (given above) under conditions identical to those illustrated in Fig. 3. The differences in control rates are due to interexperimental variability (*see text*).
* $P < 0.05$, compared to own control values.

Table 3. Arginine-vasopressin-dependent stimulation of Na/H-exchange; effect at various Na concentrations

Na concentration (mM)	*n*	Control (Δ pH/min)	AVP-treated (Δ pH/min)	% Stimulation
5.5	5	0.094 ± 0.007	0.135 ± 0.009	44.19 ± 2.65*
11	5	0.145 ± 0.013	0.177 ± 0.015	22.37 ± 3.47*
22	5	0.220 ± 0.011	0.277 ± 0.017	25.44 ± 1.93*
55	5	0.421 ± 0.041	0.502 ± 0.047	18.35 ± 2.96*
110	4	0.509 ± 0.017	0.596 ± 0.014	17.33 ± 2.21*

Experiments were performed analogous to those given in Fig. 3 and Table 2. Arginine-vasopressin concentration was 2.5×10^{-7} M, and the Na concentrations in the basolateral perfusate are given above.
* $P < 0.05$, compared to own control values.

A comparison of the extent of stimulation observed at 2.5×10^{-7} M arginine-vasopressin measured at either 22 mM Na (Fig. 3; ≈25%) or at 5.5 mM Na (Table 2; ≈44%) suggested that the arginine-vasopressin effect might depend on the degree of Na-activation of Na/H-exchange. Therefore, we have analyzed the hormone-dependent stimulation of exchange activity at a fixed arginine-vasopressin concentration (2.5×10^{-7} M), but at various Na concentrations (Table 3). Assuming a simple Michaelis-Menten relationship and using a nonlinear least-squares fit program we have derived the K_m^{Na} and the V_{max} from the data presented in Table 3. The K_m^{Na} was 44.0 ± 7.2 mM and 36.9 ± 5.9 mM for control and AVP-treated cells, respectively. The difference in V_{max} was about 10%, and the difference between both fits was statistically significant by the F test method ($0.01 \leq P \leq 0.025$). Consequently, it appears probable that at least part of the observed effect of AVP on the

Table 4. Effect of pharmacological agents on Na/H-exchange

Agonist in perfusate	*n*	Control (Δ pH/min)	Treated (Δ pH/min)	% Stimulation
Forskolin (10 μM)	4	0.223 ± 0.021	0.290 ± 0.024	30.24 ± 4.68*
8-Br-cAMP (0.1 mM)	4	0.242 ± 0.027	0.305 ± 0.026	26.70 ± 4.14*
TPA (100 nM)	5	0.213 ± 0.034	0.211 ± 0.031	

The experiment was performed in a manner identical to that given in Fig. 3. Instead of arginine-vasopressin, the agonists at the concentrations given in the figure were added.
* $P < 0.05$, compared to control values.

pH_i recovery rate can be explained by a change in Na affinity of the exchanger.

Intracellular Mediation of Vasopressin Effect

In our previous report on A6-2F3 cells we have observed a vasopressin-dependent increase in cellular cAMP-content [4]; a vasopressin-dependent increase in cellular cAMP content was also observed in A6-C1 cell monolayers, the increase being magnified (about 10–15 fold) by aldosterone pretreatment of the monolayers [25]. Previous observations on A6-cell monolayers suggested that cAMP is an important mediator of vasopressin action in A6 cells grown under similar conditions [e.g., 11, 21].

The data summarized in Table 1 document that in the A6-C1 monolayers, grown and kept under identical conditions as used in the measurements of Na/H-exchange activity, pharmacological activation of protein kinase A was able to mimic the vasopressin action on short circuit current. The experiments summarized in Table 4 extend the above data and suggest also a participation of the cAMP regulatory pathway in vasopressin stimulation of basolateral Na/H-exchange in A6-C1 cell monolayers. Na/H-exchange activity is stimulated after preincubation (10 min) of monolayers with either 10 μM forskolin or 0.1 mM 8-Br-cAMP. In contrast, the phorbolester TPA (100 nM) was unable to mimic the vasopressin-induced stimulation of Na/H-exchange activity in A6-C1 cell monolayers. It is worth mentioning that TPA did also not reproducibly affect amiloride-sensitive short circuit current (Na^+ reabsorption) (F. Verrey, *personal communication*). However, it stimulated amiloride-insensitive short circuit current (Cl^- secretion) and apical membrane movements in aldosterone-treated and -untreated A6-C1 cells ([25] and F. Verrey, *unpublished observations*). This indicates that regulation of specific transport functions in A6-C1 cells may be under the control of protein kinase C, but that in contrast to our previous report, protein kinase C activation does not mediate the AVP-dependent modulation of basolateral Na/H-exchange activity. This conclusion is also supported by the fact that AVP concentrations (10^{-10} M) that produced near maximal IP_3 accumulation and significant inhibition of Na/H-exchange in A6-2F3 cells [4] are without effect on short circuit current ($K_{0.5} = 5 \times 10^{-8}$ M; F. Verrey, *personal communication*), apical membrane movement ($K_{0.5} = 8 \times 10^{-8}$ M; ref. 25) and Na/H exchange activity (this paper). Given the dose dependences of A6-C1 cell responses to AVP, and the previous findings on dependence of apical membrane movement on levels of cAMP accumulation [25], the present findings on regulation of A6-C1 cell Na/H exchange activity are most consistent with the view that AVP actions in A6-C1 cells are mediated via cAMP.

Although it is possible that the two A6 cell preparations also express different AVP receptors, the fact that AVP stimulated cAMP production in both preparations but that forskolin and cAMP had opposite actions [4, 25] indicates that differences in the regulatory cascades downstream to the adenylate cyclase can account for the observed difference in Na/H-exchange regulation.

Conclusions

A6-C1 cell monolayers contain a basolaterally located Na/H-exchange activity. This Na/H-exchange activity is stimulated by vasopressin; pharmacological activation of the cAMP-dependent regulatory cascade mimics this regulation. A parallelism between vasopressin-dependent alteration of "physiological" function (e.g., short circuit current; mimicked by cAMP) and regulation of Na/H-exchange (mimicked by cAMP) suggests the possibility that altered Na/H-exchange function could mediate the hormonal control of the Na transport. In accordance with such a possibility are observations on the pH_i sensitivity of the apical Na permeability (higher with increasing pH_i) in tight epithelia [e.g., refs. 7, 18].

However, inhibition of the Na/H-exchanger did not prevent the stimulation of amiloride-sensitive short circuit current by AVP (*data not shown*) indicating that this stimulation was not mediated by the change in Na/H-exchanger function.

A cAMP-mediated stimulation of Na/H-exchange is not unique for the A6-C1 cell Na/H-exchanger but has also been observed for the trout erythrocyte Na/H-exchanger; unlike the recently cloned NHE1 type exchanger, the erythrocyte Na/H-exchanger has been shown to contain protein kinase A consensus sites in the C-terminal end, which are essential for cAMP activation [2, 29]. Despite there being some homology in regulation of the A6-C1 cell exchanger and the trout erythrocyte exchanger, the present data do not permit to extrapolate on the structural similarity of the A6-C1 cell exchanger. Indeed, the different response of basolaterally located Na/H-exchangers to cAMP may also be related to differences in cell-specific regulatory machinery ultimately affecting Na/H exchange activity.

The striking differences in the findings on regulatory control of basolaterally located Na/H-exchange activity in our previous work on A6 cells, studied only within a few days of reaching confluency and not pretreated with aldosterone [4], and the observations made in the present study with highly differentiated A6-C1 cell monolayers, suggest that differences in basic regulatory properties rather than the expression of different forms of Na/H-exchanger molecules are the basis of the observed differences. As multiple parameters are different between the previous [4] and the present study (e.g., clonal selection/cell population, growth condition, time of culture, filter support, collagen used) it appears difficult to find out all reasons for these differences. However, the pretreatment with aldosterone can certainly account for part of them since it has been shown that adrenal steroid hormones potentiate the action of AVP on cellular cAMP accumulation in the same A6-C1 cell preparations [25]. Indeed, we have observed that AVP administration to cells not treated with aldosterone did not reproducibly alter the Na/H-exchange activity (*data not shown*). As under the conditions used in the present study, the vasopressin-dependent alteration of physiological function (i.e., short circuit current) was always well developed, we must assume that the present observations made on A6-C1 cell monolayers are in closer correlation to "physiological" properties of (a) distal nephron epithelial cell(s) than the previously reported observations made on A6-2F3 cells not pretreated with aldosterone [4].

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