Effect of altered Na\textsuperscript{+} entry on expression of apical and basolateral transport proteins in A6 epithelia

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Lebowitz, Jonathan, Bing An, Robert S. Edinger, Mark L. Zeidel, and John P. Johnson. Effect of altered Na\textsuperscript{+} entry on expression of apical and basolateral transport proteins in A6 epithelia. Am J Physiol Renal Physiol 285:F524–F531, 2003. First published May 13, 2003; 10.1152/ajprenal.00366.2001.—In several in vivo settings, prolonged alterations in the rate of apical Na\textsuperscript{+} entry into epithelial cells alter the ability of these cells to reabsorb Na\textsuperscript{+}. We previously modeled this load dependence of transport in A6 cells by either decreasing Na\textsuperscript{+} entry via apical Na\textsuperscript{+} removal or amiloride or enhancing Na\textsuperscript{+} entry by chronic short-circuiting (Rokaw MD, Sarac E, Lechman E, West M, Angeski J, Johnson JP, and Zeidel ML. Am J Physiol Cell Physiol 270: C600–C607, 1996). Inhibition of Na\textsuperscript{+} entry by either method was associated with striking downregulation of transport rate as measured by short-circuit current (I\textsubscript{sc}), which recovered to basal levels of transport over a period of hours. Conversely, upregulation of Na\textsuperscript{+} entry by short-circuiting resulted in a sustained increase in transport rate that also returned to basal levels over a period of hours. The current studies were undertaken to determine whether these conditions were associated with alterations in either the whole cell content or apical membrane distribution of sodium channel (ENaC) subunits or on basolateral expression of either of the subunits of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. We compared these effects to those achieved by chronic upregulation of Na\textsuperscript{+} transport by aldosterone. Whole cell levels of ENaC subunits were measured by immunoblot following 18-h inhibition of Na\textsuperscript{+} entry achieved by either tetramethylammonium replacement of Na\textsuperscript{+} or apical amiloride or after an 18-h increase in Na\textsuperscript{+} entry achieved by chronic short-circuiting. None of these maneuvers significantly altered the whole cell content of any of the ENaC subunits compared with control cells. We then examined the effects of these maneuvers on apical membrane ENaC expression using domain-specific biotinylation and immunoblot. Inhibition of Na\textsuperscript{+} entry by either method was associated with a profound decrease in apical membrane α- or γ-ENaC amounts. Restoration of apical Na\textsuperscript{+} and/or removal of amiloride resulted in return of I\textsubscript{sc} to control levels over 2 h and coincided with return of apical β-ENaC to control levels without change in apical α- or γ-ENaC. Stimulation of Na\textsuperscript{+} transport by short-circuiting, in contrast, did not significantly alter apical membrane composition of any of the ENaC subunits. Basolateral expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was also measured by biotinylation and immunoblot and was unchanged under all conditions. Aldosterone increased basolateral expression of the α-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. These results suggest that chronic downregulation of transport is mediated, in part, by a selective decrease in apical membrane ENaC expression, consistent with our previous observations of noncoordinate regulation of ENaC expression under varying transport conditions in A6 cells. The chronic increase in the rate of Na\textsuperscript{+} entry is not associated with any of the changes in transporter density at either apical or basolateral membrane seen with aldosterone, suggesting that these two mechanisms of augmenting transport are completely distinct.

IT IS WELL KNOWN that changes in sodium delivery to the tubular epithelium result in changes in sodium handling along the nephron. Various conditions decrease distal sodium delivery, including volume depletion, loss of upstream glomerular filtration, direct inhibition with diuretics, and obstructive uropathy. Because the sodium delivery is diminished, all these conditions result in decreased tubular capacity to transport Na\textsuperscript{+} (8, 15, 26). In contrast, increased distal Na\textsuperscript{+} delivery as occurs in volume expansion and the administration of loop diuretics lead to hypertrophy of distal convoluted tubule and collecting duct with increased transepithelial Na\textsuperscript{+} reabsorption in the distal nephron (8, 26, 29). Additionally, acute or chronic alterations in transporter density at both apical and basolateral membranes of renal epithelia have been reported with a variety of stimuli, including chronic Na\textsuperscript{+} loading, pressure natriuresis, and hypokalemia (8, 25, 38). We developed a cell culture model to define how chronic changes in the rate of apical Na\textsuperscript{+} entry regulate transepithelial Na\textsuperscript{+} transport by inhibiting or stimulating Na\textsuperscript{+} entry for 18 h in confluent cultures of A6 cells, grown on filter-bottom supports (31). Inhibition was performed by blockade of Na\textsuperscript{+} channels with amiloride or by mole-for-mole replacement of Na\textsuperscript{+} in the apical medium with tetramethylammonium (TMA). Chronic stimulation of the Na\textsuperscript{+} channel was accomplished by 18 h of “flooding” the filter-bottom supports so that the apical media came into direct contact with the basolateral media, effectively short-circuiting the monolayers. With the use of this model, we showed that chronic inhibition of Na\textsuperscript{+} entry by either mechanism decreases both net transport rate, measured as amiloride-sensitive short-circuit current (I\textsubscript{sc}), and maximal transport rate of amiloride.
capacity, estimated by nystatin-stimulated $I_{sc}$. Conversely, chronic upregulation of Na$^{+}$ entry enhanced both basal $I_{sc}$ and nystatin-stimulated currents. Similarly, ouabain binding was decreased when apical Na$^{+}$ entry was inhibited and ouabain binding was increased when apical Na$^{+}$ entry was stimulated. This implies that at least part of the effect of altered Na$^{+}$ entry on transport rate is due to changes in Na$^{+}$-K$^{+}$-ATPase activity.

Both up- and downregulation of transport induced by alterations in Na$^{+}$ entry rate were time-dependent phenomena and required 18 h of altered Na$^{+}$ entry. Short time courses were not associated with sustained changes in transport rate (31). The effects were also time dependent in that the recovery to baseline transport took place over 1 to several hours. This observation suggested to us the possibility that chronic regulation of transport rate by the rate of Na$^{+}$ entry would be due to alteration in the density of Na$^{+}$ transport proteins, either epithelial Na channel (ENaC) or Na$^{+}$- K$^{+}$-ATPase, in the whole cell at the relevant cellular membrane. This possibility was particularly interesting as we recently examined the steady-state distribution and effects of a number of hormonal manipulations of transport rate on apical membrane expression of ENaC subunits in A6 cells (37). Our observations suggested that turnover of apical membrane subunits was somewhat variable, with β-ENaC having a shorter half-life after reaching the apical membrane than either α- or γ-ENaC. Interestingly, upregulation of transport by vasopressin or downregulation of transport by brefeldin A (BFA) was associated predominantly with changes in apical membrane β-ENaC, suggesting the possibility of noncoordinate regulation of ENaC subunit expression. Our model of chronic regulation of transport rate by rate of Na$^{+}$ entry provides us with another method of altering Na$^{+}$ transport, permitting us to examine the possibility of noncoordinate regulation of ENaC expression. Finally, it has been suggested that some of the effects of aldosterone might be mediated by chronic upregulation of Na$^{+}$ entry. We wished to compare the effects on apical and basolateral transporter density of long-term aldosterone and our model of continuous short-circuiting to determine whether there were direct effects of altered Na$^{+}$ entry in the absence of mineralocorticoid hormones.

**METHODS**

**Cell lines.** A6 cells were maintained in amphibian medium containing 10% FBS (Whitaker Bioproducts, Walkersville, MD) at 28°C in humidified air with 5% CO$_2$. Cells were seeded at high density on permeable supports (Nunc) and used at least 8 days postplating. To ensure that the cells were viable, we used an in-hod, short-circuiting device employing sterile KCl-agar salt bridges in apical and basolateral solutions to measure $I_{sc}$ as has been previously described (18, 31, 37).

**Model.** A6 cells are seeded onto porous supports (Nunc). Under these conditions, they have a high-resistance, polarized epithelium that conducts sodium in a vectorial fashion from the apical to the basolateral compartment. The tonicity of the media is 240 mosmol/kgH$_2$O of which the primary solute is Na$^{+}$. As previously described (31), sodium entry could be decreased by replacing apical Na$^{+}$ in the medium with equimolar amounts of TMA for an 18-h period. Additionally, Na$^{+}$ entry was blocked using 10 μM amiloride, a well-described inhibitor of ENaC. Alternatively, Na$^{+}$ entry could be chronically stimulated by short-circuiting, achieved by flooding the porous supports with media so that the apical and basolateral media were brought into contact. Contemporaneous controls from the same plating were treated identically except that the composition of the apical medium was not altered.

**Antibodies and reagents.** β- and γ-ENaC antibodies were generated and affinity purified as described (37). Anti-α-ENaC antibodies were obtained from Thomas Kleyman and were described and characterized by Zuckerman et al. (39). Antibodies to α- and β-subunits of Na$^{+}$-K$^{+}$-ATPase were obtained from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma unless otherwise noted.

**Quantification of whole cell ENaC.** A6 cells were grown on six-well filter inserts and subjected to control, TMA, amiloride, short-circuit, or aldosterone for an 18-h period. Cells were washed with ice-cold PBS ×5 to remove FBS and harvested by scraping. Cells were sonicated at 7.5 for 7 × 2 and protein assays were performed. For whole cell measurements, 50 μg of cell lysate were placed in sample buffer (37), and proteins separated on SDS-PAGE gels and Western blots were performed with the antibodies to ENaC subunits. Control and experimental samples for each observation were run together, transferred to nitrocellulose, and visualized by enhanced chemiluminescence as previously described (37). Samples were analyzed together to control for both sample loading and exposure time. As we previously described, the anti-α-ENaC antibody recognizes a doublet of 150, 180 kDa in Western blots from A6 cells, with typically more of the 180-kDa form in apical membrane-biotinylated samples. This antibody specifically recognizes α-ENaC expressed in vitro (39) or in HeLa cells at 75 kDa (37) and recognizes newly synthesized α-ENaC by immunoprecipitation from radiolabeled cells also at 75 kDa in A6 cells (37). Western blot analysis of cell lysates from A6 consistently give the higher molecular weight bands described above, which are competed away by preincubation with immunizing peptides (21, 37, 39). We feel that this higher molecular weight protein represents the fully mature form of α-ENaC expressed at the apical membrane in A6 (21, 37, 39). Identical high-molecular-weight bands are seen in A6 lysate using an antibody raised against a COOH-terminal epitope of α-ENaC (39) and using the α-ENaC antibody generated by Knepper and colleagues (24) and kindly provided by those investigators (not shown). Other investigators described α-ENaC as migrating at different molecular weights in A6 cells. With the use of antibodies prepared against different epitopes of the protein, Alvarez De La Rosa et al. (1) described α-ENaC as two bands appearing at 85 and 65 kDa, and Stockand et al. (34) described a protein migrating at 85–90 kDa. The antibodies to β- and γ-ENaC were generated in our laboratory and recognize bands at 97 kDa in A6 cells (37). These bands were quantitated by densitometry, and all results are expressed as a percentage of the mean values of simultaneously measured control levels.

**Quantification of apical ENaC and basolateral Na$^{+}$-K$^{+}$-ATPase.** A6 cells were grown on six-well filter inserts and subjected to control, TMA, amiloride, short-circuit, or aldosterone for 18 h. Cells were washed with ice-cold PBS with agitation at 28°C ×5 to remove FBS-containing medium. The cells were then biontynated in borate buffer on the apical surface (for quantification of membrane-bound...
ENaC subunits) or on the basolateral surface (for quantification of membrane-bound Na\(^+-\)K\(^+-\)ATPase). The nonbiotinylated side of the monolayer was bathed in medium containing FBS to prevent biotinylination. After 20 min, basolateral and apical sides were aspirated and FBS-containing medium was placed on the cells to quench the signal. Monolayers were then washed \(\times5\) with ice-cold PBS with agitation at 28\(^\circ\)C, and the cells were harvested. Cell homogenate was obtained by sonication at 7.5 for 7 s \(\times2\) in a cooling block and then centrifuged on a tabletop device \(\times5\) min at 5,000 rpm. Cell homogenate was then assayed for protein, and 300 \(\mu\)g were placed on 150 \(\mu\)L of avidin beads (37). Samples from avidin beads were collected in 2\(\times\) sample buffer and heated to 100\(^\circ\)C for 8 min to ensure complete collection. Proteins were separated by SDS-PAGE as described (37), and samples were transferred to nitrocellulose membranes and subjected to Western blot analysis with the appropriate antibodies and visualized with enhanced chemiluminescence (PerkinElmer Life Science). Simultaneous controls (untreated cells) were always separated on the same gels with experimental samples. Multiple time exposures were carried out for each blot to ensure that signals were quantified when they were increasing in the linear range. Antibodies to the three ENaC subunits were visualized at molecular weights described above. The \(\alpha\)-subunit of the Na\(^+-\)K\(^+-\)ATPase was visualized at \(-110\) kDa and the \(\beta\)-subunit at \(-50\) kDa. The results were quantified by densitometry.

RESULTS

We first examined the effect of chronic regulation of apical Na\(^+\) entry on \(I_{sc}\) and apical membrane expression of the three ENaC subunits. As shown in Fig. 1, chronic alterations in the rate of Na\(^+\) entry altered transport rates similar to our previous description (31). Chronic short-circuiting resulted in a significant increase in transport rate compared with simultaneous controls, whereas chronic inhibition of Na\(^+\) entry by either substitution of apical Na\(^+\) by TMA or by chronic exposure to apical amiloride significantly inhibited transport rate when A6 cells were initially reexposed to apical Na\(^+\). Downregulation of Na\(^+\) entry by either 18-h replacement of apical Na\(^+\) with TMA or 18 h in 10 \(\mu\)M amiloride followed by replacement with normal apical media was associated with a marked change in the apical expression of ENaC. As shown in Fig. 2, replacement of apical Na\(^+\) with TMA resulted in no change in the surface expression of either \(\alpha\)- or \(\gamma\)-ENaC but caused a large and significant decrease selectively in apical membrane \(\beta\)-ENaC expression. Treatment of A6 cells for 18 h with 10 \(\mu\)M amiloride (Fig. 3) also caused a significant decrease in apical \(\beta\)-ENaC expression compared with control cells. In this case, there was also some decrease in the apical expression of \(\alpha\)- and \(\gamma\)-subunits, although these decreases did not reach statistical significance. Both downregulation conditions therefore were associated with selective decreases in apical membrane \(\beta\)-ENaC expression, similar to the effect previously noted by us when the transport rate was downregulated by BFA (37).

If the decrease in apical \(\beta\)-ENaC was of significance in the decline in \(I_{sc}\), then a minimum expectation would be that a return of \(I_{sc}\) to control levels following removal of amiloride or restoration of apical Na\(^+\) would be accompanied by a return of apical \(\beta\)-ENaC amounts to control levels. We therefore examined the effect of recovery from inhibition of apical Na\(^+\) entry on the apical expression of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC. Cells were exposed to 18-h downregulation of Na\(^+\) entry by either apical amiloride or replacement of apical Na\(^+\) as described above. After restoration of normal apical media, \(I_{sc}\) was monitored for return of ENaC function.

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**Fig. 1.** Effect of altered conditions of apical Na\(^+\) entry on short-circuit current (\(I_{sc}\)) in A6 epithelia. A6 cells grown on filter supports were either short-circuited (SC) for 18 h by flooding filters so that apical and basolateral media were in contact, exposed to 10 \(\mu\)M amiloride on the apical surface for 18 h, or had total replacement of apical Na\(^+\) by tetramethylammonium (TMA) for 18 h. After each treatment, cells were washed 3 times in regular media, \(I_{sc}\) was measured within 5–10 min and compared with simultaneous controls. All conditions are significantly different from control, \(P < 0.01\); \(n = 8–12\).

**Fig. 2.** Effect of inhibition of apical Na\(^+\) entry by TMA substitution on apical membrane expression of epithelial Na channel (ENaC) subunits. Na\(^+\) entry was blocked by 18-h substitution of TMA for Na\(^+\). After 18 h under these conditions, cells were returned to control conditions and apical membrane proteins were isolated by domain-specific biotinylination as described. Results are the mean of 6 observations each. TMA resulted in a significant decrease in apical membrane \(\beta\)-ENaC alone. Results are expressed as percentage of control mean ENaC densitometry. Top: representative immunoblots for each subunit. Molecular weights are given in METHODS. *Significantly different from control, \(P < 0.05\).
compared with control, untreated monolayers. The transport rate recovered 70% in 1 h and 100% in 2 h, consistent with our initial observations using this model (31). At the time of full recovery, control and both amiloride/recovery and TMA/recovery samples were subjected to apical membrane biotinylation as described to measure apical expression of each ENaC subunit. As shown in Fig. 4, recovery of $I_{sc}$ to control levels following removal of inhibition of apical Na$^+$ entry was associated with return of apical membrane $\beta$-ENaC to control levels.

In contrast to the remarkable effects of downregulation of transport on apical membrane ENaC expression, upregulation of transport by 18 h of short-circuiting had no substantial effect on the apical membrane expression of any of the three ENaC subunits (Fig. 5). There was a slight tendency toward an increase in apical $\beta$-ENaC expression, but this did not reach significance.

Because apical membrane ENaC expression had been altered by chronic downregulation of Na$^+$ transport, we next sought to determine whether this was a reflection of altered whole cell amounts of ENaC subunits or alternatively whether it might represent a redistribution of ENaC subunits in response to the altered Na$^+$ entry. Whole cell ENaC subunit densities under the two conditions are shown in Fig. 6. Replacement of apical Na$^+$ with TMA selectively reduced apical membrane $\beta$-ENaC but only reduced $\gamma$-ENaC in whole cells. Blockade of apical Na$^+$ entry with 18 h of 10 $\mu$M amiloride significantly and markedly reduced apical membrane-associated $\beta$-ENaC with slight but not significant changes in the other subunits but had no significant effect on the whole cell amounts of either $\alpha$- or $\beta$-ENaC.

Because alterations in the rate of Na$^+$ entry were associated with significant changes in the activity of Na$^+$-K$^+$-ATPase activity that persisted after return to normal growth conditions in our initial study (31), we...
Also examined the effect of these maneuvers on the basolateral membrane expression of \( \alpha \)- and \( \beta \)-subunits of the ATPase. As shown in Fig. 7, neither chronic upregulation of transport rate by short-circuiting nor chronic downregulation of transport by TMA substitution for apical Na\(^+\) had any effect on the amount of either \( \alpha \)- or \( \beta \)-subunit of the Na\(^+\)-K\(^+\)-ATPase present in the basolateral membrane.

Although we were unable to demonstrate any effect of chronic upregulation of Na\(^+\) entry on the expression of apical or basolateral transport proteins, we previously demonstrated that long-term (18 h) but not short-term (3 h) exposure of A6 cells to 10\(^{-6}\) M aldosterone results in a selective increase in apical membrane expression of \( \beta \)-ENaC (37). This occurred without an effect on the apical membrane expression of either \( \alpha \)- or \( \gamma \)-ENaC, the mirror image of what we described with downregulation of Na\(^+\) entry by TMA substitution. A similar change in whole cell amounts of \( \beta \)-ENaC expression without a change in \( \alpha \)- or \( \gamma \)-ENaC has been described by Stockand and colleagues (34) following long-term aldosterone exposure. Although we were unable to demonstrate changes in basolateral Na\(^+\)-K\(^+\)-ATPase amounts following alteration in Na\(^+\) entry, the effects of aldosterone on both transcription and translation of both subunits of this enzyme are well described (2, 36). Additionally, we previously demonstrated upregulation of enzyme activity in A6 cells, which is unaffected by blockade of Na\(^+\) entry with amiloride (18). Figure 7 demonstrates the effect of 18-h aldosterone treatment on the basolateral membrane amounts of \( \alpha \)- and \( \beta \)-subunits of Na\(^+\)-K\(^+\)-ATPase. Aldosterone significantly upregulates the amount of the \( \alpha \)-subunit in basolateral membrane without affecting basolateral amount of \( \beta \)-subunit.

**DISCUSSION**

It has been shown in a number of mammalian models that chronic alterations in delivery or chronic blockade of specific Na\(^+\) transporters may induce adaptive changes in transport rates that appear to be dependent on load (8, 15, 27, 29, 38). In in vitro systems, acute stimulation of Na\(^+\) entry results in reduction of ENaC activity through a process of feedback inhibition (16, 19, 30), whereas acute inhibition of Na\(^+\) entry is associated with increased ENaC activity (11, 30). Because chronic increases in Na\(^+\) load or delivery in animals appear to be associated with transporter upregulation (8, 27), whereas chronic inhibition of transport seems to be associated with downregulation of transporters (15), we examined the long-term effects of these maneuvers in vitro. We established a model of chronic regulation of Na\(^+\) transport by the rate of apical Na\(^+\) entry in the cultured A6 cell line, where Na\(^+\) transport is primarily mediated by apical membrane ENaC and the basolateral Na\(^+\)-K\(^+\)-ATPase (31). In this model, chronic upregulation of Na\(^+\) entry by continuous short-circuiting of the epithelia for 18 h resulted in upregulation of transport rate when normal polarity was restored. Transport rates declined toward basal levels over a period of hours. In contrast, chronic downregulation of apical Na\(^+\) entry by either removal of apical Na\(^+\) or ENaC blockade with amiloride for 18 h resulted in a decreased rate of transport. When normal apical Na\(^+\) entry was restored, \( I_{sc} \) returned to control levels within 2 h. In contrast to our results, studies in primary
cultures derived from the rabbit kidney collecting system showed little effect of chronic short-circuiting and chronic inhibition of Na\(^+\) entry with benzamil-enhanced transport rates (6). In these experiments, chronic short-circuiting, which had little effect on either basal \(I_{sc}\) or \(\beta\)-ENaC levels, blocked the effect of aldosterone and this inhibition was overcome by incubation with benzamil (6). Noting the difference between their results and our initial studies (31), the authors speculated that intracellular Na\(^+\) concentration or an alteration in rates of Na\(^+\) entry might have dual effects, depending on the absence or presence of aldosterone. The current experiments were designed to reevaluate our model of chronic regulation and test the possibility that chronic alterations in Na\(^+\) entry altered channel subunit expression or basolateral enzyme expression under conditions of chronic up- or downregulation. Additionally, we sought to compare the effects of chronic upregulation by increased Na\(^+\) entry to those seen with chronic upregulation of transport by aldosterone.

Our results confirmed some but not all of our expectations. Chronic downregulation of Na\(^+\) entry by either mechanism was associated with decreased expression of ENaC subunits at the apical membrane without significant changes in whole cell subunit content. Of particular interest, only one subunit, \(\beta\)-ENaC, was decreased in apical expression. This observation is similar to what we described with downregulation of transport by the agent BFA (37). BFA disrupts delivery of proteins to post-Golgi targets and markedly reduced \(I_{sc}\) in A6 cells but resulted in a decrease in apical expression only of \(\beta\)-ENaC with no significant change in \(\alpha\)- or \(\gamma\)-expression. This occurred over a period of 1- to 3-h incubation with BFA, similar to the time course of BFA inhibition of apical membrane channel activity measured by noise analysis (9). This surprising result was explained to some degree when we examined the half-life of ENaC subunits that reach the apical membrane in A6 (37). \(\beta\)-ENaC turns over with a half-life of several hours, whereas \(\alpha\)- and \(\gamma\)-ENaC that reach the apical membrane appear to be considerably more long lived (21, 37). We referred to this phenomenon of apparent differential turnover of apical membrane ENaC subunits as noncoordinate regulation. It appears that downregulation of Na\(^+\) entry in A6 cells may be another example of this.

Because there is no reason to believe that physiologically significant ENaC function is mediated by anything other than fully formed heterotrimERIC channels, the observation of noncoordinate regulation implies that channels may be assembling or disassembling at some point beyond the endoplasmic reticulum (ER). As Rotin and colleagues (32) recently pointed out, there is no evidence to support such a concept that emerges from the many studies of ENaC assembly or function in oocytes or heterologous expression systems, which clearly demonstrate that ENaC assembles into complete tetrameric channels in ER soon after biosynthesis as do most multimeric proteins. It is interesting to note, however, that multimeric proteins may be processed differently in endogenously expressing cells than in overexpressing cells. The T cell antigen receptor complex (TCR-CD3) of T cell hybridomas has served as an established model of ER assembly of a multimeric membrane protein, but when examined in normal T cells, the putative limiting \(\zeta\)-subunit appears to exhibit more rapid turnover from membrane-bound complexes than do the other subunits (28). Similarly, the three subunits of the interleukin-2 receptor in T lymphocytes appear to have varying surface half-lives and endocytic fates (14). In cells or tissues endogenously expressing ENaC, there are multiple examples of apparent noncoordinate regulation of individual subunits. In rat distal nephron, aldosterone induces expression of \(\alpha\)-ENaC with an apparent shift of cytosolic \(\beta\)- and \(\gamma\)-subunits to the apical membrane with a short course in early distal nephron and a more prolonged course in collecting duct (10, 23, 24). Physiological manipulations to rats have been described that result in either selective regulation of \(\beta\)- and \(\gamma\)-ENaC (7, 13, 20) or of \(\beta\) alone (3). Selective upregulation of \(\beta\)- and \(\gamma\)-ENaC has also been described to alter the biophysical properties of ENaC expressed in alveolar cells (22), and long-term PKC stimulation inhibits the transport rate in A6 epithelia in association with a selective decrease in \(\beta\)- and \(\gamma\)-ENaC without changes in \(\alpha\)-ENaC (34). Overexpression of \(\beta\)-ENaC in A6 cells results in enhanced transport rates (4), but surface subunit amounts were not measured in this study. The precise mechanism by which noncoordinate regulation of ENaC subunit expression results in changes in the apical density of fully active channels is unclear, but in each case, regulation of the transport rate appears to correspond with altered expression of some, but not all, ENaC subunits (3, 7, 13, 20, 22–24, 34, 37). A recent report by Alvarez De La Rosa et al. (1), which describes fully coordinate expression and turnover of cellular and apical membrane ENaC subunits in response to aldosterone in A6 cells, clearly contradicts the notion of noncoordinate regulation. The reasons for the differences in results between this study and our own previous findings as well as those of other groups (10, 29, 32) are not clear, because many of the same methods were employed. Alvarez De La Rosa et al. (1) describe an extremely short half-life (12–17 min) for ENaC subunits that reach apical membrane, a period in marked contrast to the longer half-life we (37) and others (21) described in A6 cells and difficult to reconcile with the time course of BFA inhibition of ENaC surface density measured by noise analysis (9).

In contrast to our expected finding of ENaC downregulation by the rate of Na\(^+\) entry, we could find no evidence of a change in apical membrane ENaC expression with chronic short-circuiting and no evidence of a change in basolateral Na\(^+\)-K\(^+\)-ATPase by either chronic adaptation. Clearly, altered enzyme activity in chronic adaptation is not a function of a greater or lesser number of pumps expressed in the membrane. Although we could measure no direct effects of upregulation of Na\(^+\) entry by short-circuiting on apical or basolateral transporter density, chronic upregulation...
of transport by aldosterone clearly affected both. Once again, the characteristic of this regulation was noncoordinate both with respect to ENaC and ATPase. Selective upregulation of apical β-ENaC by aldosterone in A6 has previously been noted (37), and data from Stockand et al. (34) suggest this is due to increased expression of this subunit at the whole cell level. Both transcriptional and translational regulation of both subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by aldosterone have been well documented previously (2, 17, 29, 36). A recent observation in cultured cortical collecting duct cells demonstrates a roughly similar increase in basolateral α-subunit to that which we describe here, but no data on β-subunit expression at basolateral membrane are available (35). Previous studies suggest that the two subunits may have slightly different time courses of membrane insertion (5, 12), and relative differences in fold-changes in the two subunits expression following regulatory stimuli have been described (25, 38). These observations suggest that the enzyme may not traffic exclusively as αβ-heterodimers. Moreover, it has been shown that increases in a single subunit of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase not only occur but have also been implicated in increasing pump activity (33). Our observations would suggest that enzyme function may be increased by alteration in basolateral expression of α-subunit alone, which implies that there may be an excess of β-subunit already present in or near the membrane. Whatever the mechanism, aldosterone clearly induces alterations in apical ENaC and basolateral Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, which are not seen with chronic upregulation of Na\textsuperscript{+} entry alone. Our data are in agreement with the conclusion by Summa and colleagues (35) that the effect of aldosterone on basolateral transport proteins is not mediated by apical Na\textsuperscript{+} entry.

DISCLOSURES

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