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Noncoordinate regulation of ENaC: paradigm lost?

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Weisz, Ora A. and John P. Johnson. Noncoordinate regulation of ENaC: paradigm lost? *Am J Physiol Renal Physiol* 285: F833–F842, 2003; 10.1152/ajprenal.00088.2003.—The epithelial sodium channel (ENaC) is composed of the three homologous subunits α, β, and γ. The basic oligomerization process inferred from all studies in heterologous systems is preferential assembly of the three subunits into a single oligomeric form. However, there is also considerable evidence that channels composed of only α-, αβ-, or αγ-subunits can form under some circumstances and that individual subunits expressed in heterologous systems can traffic to the cell membrane. In cells that express endogenous ENaC, the three subunits are often synthesized in a differential fashion, with one or two subunits expressed constitutively while the other(s) are induced by different physiological stimuli in parallel with increased ENaC activity. This phenomenon, which we term noncoordinate regulation, has been observed for both whole cell and apical membrane ENaC subunit expression. Several other heteromeric membrane proteins have also been observed to have differential rates of either turnover or trafficking of individual subunits after biosynthesis and membrane localization. Here, we examine the possibility that noncoordinate regulation of ENaC subunits may represent another mechanism in the arsenal of physiological responses to diverse stimuli.

epithelial sodium channel; trafficking; aldosterone; T cell antigen receptor; assembly

THE KIDNEY REGULATES EXTRACELLULAR fluid volume in the body through modulation of Na^+ reabsorption along the nephron. The ultimate regulation of Na^+ reabsorption occurs in the collecting ducts via a process of conductive transport mediated by the epithelial sodium channel (ENaC). This channel is located in the apical membrane of collecting duct principal cells and represents the rate-limiting step in Na^+ reabsorption in this segment. ENaC is also located in the apical membrane of other epithelial tissues throughout the body, including colon, sweat glands, and airway (3, 31). Abnormalities of function of this channel, linked to inherited alterations in channel sequence, have been shown to be important in several human diseases, including the hypertension seen in patients with Liddle's syndrome (73) and the salt-wasting seen with some variants of psuedohypoaldosteronism (15). In addition, apparent overactivity of this channel in patients with cystic fibrosis has been linked to the pathogenesis of airway disease (10). These effects observed in genetic diseases may point to a significant impact of Na^+ channel dysregulation in volume-overload states such as congestive heart failure, liver failure, and nephrosis as well as hypertension.

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The primary structure of ENaC was elucidated through expression cloning (14) and revealed that the channel is formed by three homologous subunits, α, β, and γ. When these three subunits are expressed together in *Xenopus laevis* oocytes, they produce a channel with the typical biophysical and pharmacological properties of the native channel: low conductance, high Na^+/K^+ selectivity, and sensitivity to amiloride in the submicromolar range (31). In endogenously expressing tissues, ENaC is present in relatively small copy number at apical membranes, so many studies of assembly and trafficking, as well as structure-function studies, have been carried out in overexpression systems. However, the picture that emerges from these studies is not always concordant with observations in endogenously expressing cells. The basic observations on trafficking and stability of ENaC have recently been reviewed (70) and will be briefly summarized below. We will then review observations from other systems that raise questions concerning the universality of this paradigm and discuss the phenomenon of noncoordinate regulation of heteromeric membrane proteins.

ASSEMBLY AND TRAFFICKING OF ENaC IN HETEROLOGOUS EXPRESSION SYSTEMS

The biosynthesis of individual ENaC subunits has been examined in detail in several heterologous expression systems. The general consensus that has emerged from these studies is that ENaC assembly is

inefficient and that the majority of newly synthesized ENaC subunits are rapidly degraded. In *X. laevis* oocytes, the proteasome is largely responsible for degrading ENaC as inclusion of the proteasome inhibitor lactacystin dramatically increases the half-lives of individually expressed rat ENaC subunits (78). When all three subunits are coexpressed, proteasomal degradation is decreased, and the half-life of each subunit increases from ~4 to ~10 h; thus assembly of subunits into oligomeric channels results in their stabilization (74, 78). Short half-lives (1–2 h) for newly synthesized ENaC subunit pools were also observed in mammalian cell lines (74).

ENaC Assembly and Cell Surface Expression

The efficiency of assembly and trafficking of ENaC subunits to the cell surface in heterologous expression systems is more controversial. Studies in heterologous expression systems (1, 18) and oocytes (27, 46) suggest that the channel subunits oligomerize with some fixed stoichiometry soon after synthesis in the endoplasmic reticulum (ER). Early oligomerization of ENaC subunits has been observed in COS cells and in vitro translation systems, as demonstrated by coimmunoprecipitation of the three subunits in both glycosylated and nonglycosylated forms (18).

The efficiency of ENaC trafficking to the cell surface is widely variant among studies and may be related to the level of expression. In oocytes, only a small fraction (<1%) of total ENaC channels are present at the plasma membrane at steady state (78), and Hanwell et al. (33) observed similar results in stably expressing Madin-Darby canine kidney (MDCK) cells. In the latter study, the half-life of ENaC subunits at the cell surface was ~1 h, comparable to that of the whole cell population. These findings are consistent with electrophysiological measurements suggesting rapid turnover of cell surface ENaC in oocytes (72, 74). By contrast, Prince and Welsh (68) reported that in COS and HEK-293 cells, individual ENaC subunits traffic to the plasma membrane with high efficiency, although the cell surface subunits appear to be deglycosylated and present in detergent-insoluble complexes. In addition, at steady state, ~10–20% of subunits are localized to the plasma membrane in stably-transfected MDCK and Chinese hamster ovary cells (Hughey RP, Bruns JB, Mueller G, and Kleyman TR, personal communication). However, the half-lives of cell surface subunits were not examined in these studies.

Detailed studies of the maturation of ENaC complexes have been somewhat hampered by the apparent absence of oligosaccharide processing of the subunits in most cell types (33, 70). Recently, however, two groups have observed that the oligosaccharides on a small fraction of subunits become resistant to endoglycosidase H in some cell lines, including endogenously expressing *X. laevis* renal epithelial A6 cells (Ref. 4 and Hughey RP, Bruns JB, Mueller G, and Kleyman TR, personal communication). Resistance to cleavage by this enzyme reflects the processing of core oligosaccharides to precursors of their complex forms in the *cis*/medial-Golgi and is a useful marker by which to distinguish ENaC subunits that have reached later compartments of the biosynthetic pathway. Interestingly, however, a considerable fraction of cell surface ENaC is endoglycosidase H sensitive in both heterologous expression systems and in cells that express endogenous ENaC, suggesting that oligosaccharide processing of these proteins is unusually inefficient compared with that of other plasma membrane residents (4, 33, 70, 81).

ENaC Stoichiometry in Heterologous Expression Systems

While various stoichiometries of the subunits have been described in terms of the ultimate channel expressed in membranes, the emerging consensus is that the stoichiometry in oocytes is a heterotetramer consisting of two α-, one β-, and one γ-ENaC subunits (27). However, a stoichiometry of three α-, three β-, and three γ-subunits has also been described for this channel (71). Although ENaC channels composed of all three subunits appear to be the predominant species expressed at the oocyte plasma membrane, other combinations are possible. It is known, for example, that the α-subunit alone is capable of forming a channel (13) and that channels composed of only α- and β- or α- and γ-subunits can be expressed in oocytes; these channels demonstrate modest differences in ion selectivity, amiloride sensitivity, and open probability compared with channels that contain all three subunits. For example, αβ-channels have larger Na^+ currents than Li^+ currents, whereas αγ-channels have smaller Na^+ currents than Li^+ currents (58). Channels composed of αβ- or αγ-subunits generate only 15–20% of the current seen when all three subunits are expressed (58). This decreased current could reflect altered assembly, trafficking, and/or conductance of αβ- or αγ-channels relative to the αβγ-holochannel. Indeed, Konstas and Korbmacher (45) recently observed that αγ-channels traffic to the plasma membrane of oocytes more efficiently than αβ-channels, although both of these are more poorly expressed at the cell surface than αβγ-channels. Welsh and colleagues (1, 68) have reported that individual subunits of human ENaC, when expressed independently in cells, can also oligomerize into homomultimers that efficiently traffic to the cell surface. There is evidence that such alternative channel stoichiometries may exist in endogenously expressing cells, although they have not been demonstrated directly. For example, the recent observations that unlike α-ENaC-deficient mice, β- and γ-ENaC-deficient mice do not die due to failure to clear lung liquid at birth suggest that αβ- and αγ-channels may have sufficient activity for pulmonary clearance (36).

To summarize the insights from studies of heterologous systems, ENaC appears to assemble into a heterotrimeric complex in the ER, and a majority of synthesized subunits are degraded and never form functional surface channels. Channels made up of a single subunit or two subunits may form, but the efficiency of

their maturation and their functional significance are unclear. Assembly of the αβγ-complex appears to increase the stability of ENaC subunits, but at least in oocytes ENaC that reaches the plasma membrane appears to have a short half-life. It is retrieved from the membrane by Nedd4-mediated ubiquitination and probably also by clathrin-mediated endocytosis (41, 70, 72). It is not known whether internalized ENaC is recycled to the plasma membrane.

NONCOORDINATE REGULATION OF ENDOGENOUS ENaC IN TISSUES

The term noncoordinate regulation was first used in reference to ENaC by Farman and colleagues (25, 26) when describing distinct responses of subunit mRNA in kidney cortex, colon, and lung to stimulation by steroid hormones. Similar to earlier observations made by other laboratories, it was noted that only α-ENaC mRNA was increased by steroids in kidney, whereas β- and γ- but not α-ENaC message were increased in colon (5, 25, 26, 69). In endometrial epithelium, steroids increased ENaC activity but upregulation of only γ-ENaC mRNA was observed (77). Thus there appears to be unexpected complexity in the regulation of ENaC expression. In fact, there is considerable variability in expression and regulation of mRNA for ENaC subunits across epithelial tissues that express the channel. The consequence of noncoordinate regulation of message on channel stoichiometry and activity in epithelial tissues has not yet been examined in detail. Increases in individual subunit mRNAs might simply reflect the variety of physiologically relevant transcriptional regulators of ENaC and may not lead to changes in ENaC levels, subunit composition, or activity at the plasma membrane. However, in oocytes at least, Firsov and colleagues (27) demonstrated that a fixed stoichiometry is preferred when all three ENaC subunits are expressed, regardless of their individual levels, suggesting that noncoordinate expression of individual subunits does not alter the composition of the surface channel in this system.

Rather more surprising, and somewhat more difficult to explain using the straightforward paradigm outlined above, are the numerous observations of noncoordinate expression and regulation of individual subunit protein expression in virtually all ENaC-expressing tissues that have so far been examined. Studies examining ENaC subunit expression in response to a variety of physiological or pharmacological stimuli are summarized in Table 1. The effects of a wide variety of stimuli on renal ENaC expression have been reported, including salt and water deprivation or loading, acid or base loading, diabetes, obesity, steroid or vasopressin infusion, K^+ depletion, angiotensin receptor knockout, and chronic diuretic infusion. In most cases, there is a selective change in the levels of one or two subunits of ENaC, but rarely in all three. Moreover, in each case, the change in Na^+ transport rate corresponds to the direction of change of one or two ENaC subunits, but not all three. From immunohistochemical studies, it appears that α-ENaC is predominantly located at the apical membrane in mammalian cortical collecting duct (CCD), whereas β- and γ-ENaC are located diffusely throughout the cytoplasm and tend to relocate toward the apical membrane on stimulation of transport (32, 50, 53, 63). Intracellular vesicles that harbor these cellular stores of β- and γ-ENaC in CCD have not yet been identified or characterized (32).

NONCOORDINATE REGULATION OF ENDOGENOUS ENaC IN CULTURED CELLS

More detailed studies of ENaC trafficking and surface expression have been carried out in a number of cultured cell lines derived from renal or pulmonary tissues and have led to observations that are at some variance with those made using overexpression systems (Table 2). Studies of endogenous ENaC regulation in A6 cells have been carried out by several groups.

Table 1. *Regulation of ENaC subunit expression in animal studies*

Tissue	Physiological Change	Result	Ref. No(s).
Rat kidney and mouse kidney CCD, CNT, DT	Na^+ restriction long-term/aldosterone infusion	↑ α-ENaC, no change in β- or γ- ENaC levels, but redistribution time and site dependent	50, 51, 53, 54
Rat kidney CCD	Na^+ restriction 15–24 h	No change in levels ↓ MW γ-ENaC	29
Rat kidney CCD	Na^+ restriction, sprionolactone, long-term	Spironolactone blocks ↑ in α-ENaC, not redistribution	63
Rat kidney A-II knockout	Na^+ restriction or loading, long-term	↓ α-ENaC, ↑ β-, and γ-ENaC (restriction)	12
Rat kidney CCD, diabetic	Compared with nondiabetic	↓ α-ENaC, ↑ β-ENaC, no change in γ-ENaC	7
Rat kidney CCD, obese	Compared with lean	↑ β-ENaC, no change in α- or γ-ENaC	8
Rat kidney CCD	Acid load	↓ β- and γ-ENaC	42
	Base load	↑ β- and γ-ENaC	
Rat kidney CCD	Water restriction, long-term	↑ β- and γ-ENaC	22
Brattleboro rat CCD	AVP infusion, chronic	Marked ↑ β- and γ-ENaC, modest ↑ α-ENaC	11, 22
Rabbit kidney	Aldosterone, 6–16 h	↑ α- and β-ENaC, no change in γ-ENaC	20
Rat kidney	Chronic thiazide or loop diuretic infusion	↑ β-ENaC in cortex, ↑ β- and γ- ENaC in medulla with thiazide infusion	61
Rat kidney	K^+ depletion	↓ α-, β-, and γ-ENaC in cortex	24
Rat kidney	Water loading and AVP infusion	↑ α- and 70-kDa γ-ENaC, no change in β-ENaC	23

ENaC, epithelial Na^+ channel. ↑, Upregulation; ↓, downregulation; CCD, cortical collecting duct; CNT, connecting tubule; DT, distal tubule.

Table 2. *Regulation of ENaC subunit expression in cultured cells*

Cell Line	Physiological Change	Result	Ref. No.
A6	Aldosterone	No change at 3 h. Late ↑ α- then β-ENaC, no change in γ-ENaC	57
A6	Aldosterone	No change at 3 h. ↑ β-ENaC, 18 h in whole cell and apical membrane	81
A6	AVP	↑ β-ENaC, apical membrane, no change in α- or γ-ENaC	81
A6	Brefeldin A, 3 h	↓ β-ENaC, apical membrane, no change in α- or γ-ENaC	81
A6	Aldosterone, 24 h	↑ β-ENaC in whole cell, no change in α- or γ-ENaC	75
A6	PMA long-term	↓ γ- then β-ENaC, no change in α-ENaC	75
A6	Aldosterone, 24 h	No ↑ in α-ENaC	44
A6	Aldosterone	Progressive ↑ in whole cell and apical α-, β-, and γ-ENaC from 1 h	4
Mouse CCD cells	AVP	↑ β- and γ-ENaC, no change in α-ENaC, ↑ phenamiloride binding	21
Rat alveolar type II cells	Hypoxia	↓ Apical β- and γ-ENaC	67
	Hypoxia + β-agonist	↑ Apical β- and γ-ENaC	
A549 cells	Dexamethasone long-term, 24–48 h	↑ β- and γ-ENaC no change in α-ENaC*	48
H441 cells	Dexamethasone, 24 h	↑ α-ENaC, β- and γ-ENaC not measured	38
Fetal rat distal lung epithelial cells	Hypoxia + corticosterone	↓ α-, β-, and γ-ENaC	76

These studies are of particular interest because they sometimes include direct measurements of the apical membrane pool of ENaC subunits as well as whole cell measurements. However, there is significant conflict between the observations of several groups using A6 cells. May and colleagues (57) examined the effect of aldosterone on mRNA levels, rate of synthesis, and half-lives of the three ENaC subunits. They described a delayed effect of aldosterone (6–24 h) on mRNA levels of all three subunits, with an increase in the synthesis of α-ENaC seen after 1 h, in β-ENaC after 6 h, and no effect on γ-ENaC message. The half-lives of all three ENaC protein subunits were short (40–50 min). These data were interpreted as consistent with a model where α-ENaC synthesis was rate limiting for assembly and expression of functional channels. We have examined the expression of ENaC in both whole cells and in apical membranes under a variety of transport conditions (81). In agreement with May et al. (57) and with studies in heterologous expression systems, we measured a relatively short half-life for newly synthesized ENaC subunits (~2 h). Approximately 10–20% of the whole cell content of each subunit was expressed on the apical membrane at steady state, significantly more than the estimates of relative surface expression in oocytes and some mammalian cells (33, 78) but consistent with other studies (Ref. 68 and Hughey RP, Bruns JB, Mueller G, and Kleyman TR, personal communication). Using a surface biotinylation approach, we determined that the half-lives of those subunits that actually reached the apical membrane were significantly longer than those observed for newly synthesized subunits. Interestingly, however, the apparent half-lives of apical ENaC subunits were not all the same. Apical membrane β-ENaC had a significantly shorter half-life (5–6 h) than did apical α- and γ-ENaC (>24 h) (81). Kleyman et al. (44) have also measured a long half-life (24–30 h) for cell surface α-ENaC in A6 cells. Notably, all of these values are considerably higher than the 1-h half-life reported by Hanwell et al. (33) for ENaC subunits expressed in MDCK cells.

In addition to its relatively shorter half-life compared with other cell surface subunits, we also observed preferential redistribution of β-ENaC in response to general perturbants of membrane traffic and to physiological stimuli. When A6 cells were exposed to brefeldin A, a fungal metabolite that inhibits protein delivery to the apical membrane, there was a selective loss of cell surface β-ENaC after 3 h and no change in apical α- or γ-ENaC (81). The decrease in cell surface β-ENaC paralleled the decline in amiloride-sensitive current that occurred over this time period, which has previously been shown by noise analysis to be due to a decrease in functional channels in the apical membrane (28). We also observed a selective increase in apical β-ENaC levels that mirrored the increase in amiloride-sensitive current induced by long-term treatment with aldosterone (18 h) or by addition of vasopressin (30 min). The increase in apical membrane β-ENaC seen in response to vasopressin stimulation was accompanied by a selective decrease in β-ENaC recovered from endosome-enriched fractions on sucrose gradients (81). In contrast, treatment with insulin or incubation with aldosterone for shorter periods (3 h) increased the amiloride-sensitive current with no effect on cell surface ENaC subunit levels (81). In agreement with these results, Kleyman and colleagues (44) also found no change in apical membrane α-ENaC levels in response to overnight treatment with aldosterone.

Further evidence for noncoordinate ENaC subunit expression comes from the studies of Stockand et al. (75), who demonstrated a selective increase in cellular β-ENaC after long-term aldosterone treatment in A6 cells and a selective downregulation of β- and γ-ENaC (consistent with the decline in amiloride-sensitive current) in response to PKC activation. Downregulation of β- and γ-ENaC occurred with differing time courses, and the temporal recovery of the transport rate corresponded with the restoration of β- and γ-ENaC to their original levels (75). The effect of PKC on ENaC subunits appears to be related to targeted degradation of β- and γ-ENaC. Booth and Stockand (9) have described

a complex time course of γ-ENaC degradation in A6 cells, with the bulk of whole cell γ-ENaC degrading within 1 h and a sizable "protected" pool still detectable after 12 h. Because the PKC effect was primarily seen in this protected pool, the authors suggested that the longer-lived pool represents recycling γ-ENaC.

In striking contrast to these studies, Alvarez de la Rosa et al. (4) have made remarkably different observations on the synthesis, trafficking, and expression of ENaC in A6 cells. The half-life of the total cellular pool of ENaC subunits was short (40–70 min) consistent with numerous previous observations. In this study, however, the half-life of all ENaC subunits that reached the apical membrane was extremely short (12–17 min). These are a remarkably rapid turnover rates for plasma membrane proteins and are considerably shorter than that the half-lives we and others measured for ENaC subunits (44, 81). On treatment of cells with aldosterone, Alvarez de la Rosa et al. (4) noted an early and coordinate increase in mRNA for all three subunits in A6 cells that resulted in a progressive, coordinate increase in whole cell and apical membrane expression of the three ENaC subunits. This is the first study to demonstrate coordinate regulation of the three ENaC subunits in response to steroid hormone stimulation and contrasts with the earlier observations by May et al. (57).

It is not clear why the various studies in A6 cells show such conflicting results because they generally involve the same methods of quantitation by Northern blot analysis and Western blotting of apically biotinylated ENaC subunits. It is possible that different subclones of A6 cells have adapted distinct responses to regulating ENaC surface expression (4, 81). Another confounding issue is the unresolved molecular weight of α-ENaC in A6 cells, as anti-α-ENaC antibodies made by different laboratories recognize bands of different molecular weights on SDS-PAGE (4, 44, 75, 81). This cannot explain all of the differences between the various studies, because all groups utilized anti-β- and γ-ENaC antibodies that recognize proteins of similar molecular weights, yet arrived at different conclusions regarding the effect of aldosterone on whole cell subunit levels (4, 75, 81).

Studies in lung-derived epithelia expressing ENaC have also been associated with considerable controversy regarding the possibility that noncoordinate regulation results in the expression of phenotypically distinct channels, depending on culture conditions and hormonal stimulation (39, 55, 56). On the basis of studies in alveolar type II cells in culture, Eaton and colleagues (39, 55) have proposed that channels assembled from different combinations of ENaC subunits could give rise to functional channels of widely varying biophysical characteristics. This group has examined ENaC expression in A549 cells, a line derived from a human alveolar carcinoma that has characteristics of alveolar type II cells. Under basal conditions, they described a moderately selective and amiloride-sensitive Na^+ channel with a conductance of 8 pS (47). Stimulation of Na^+ transport by dexamethasone resulted in noncoordinate changes in ENaC subunit mRNA and protein (48). Message levels for β- and γ-ENaC were increased in the presence of dexamethasone, with a ~10-fold increase in γ-ENaC mRNA relative to β-ENaC. This was associated with a marked increase in cellular β- and γ-ENaC subunits levels with no change in α-ENaC. At the single-channel level, treatment with dexamethasone resulted in increased amiloride sensitivity, increased open probability, and a decrease in channel conductance to 4.4 pS (48). The authors suggested that their results could have resulted from alterations in the stoichiometry of the ENaC subunits. These findings have been challenged by Itani et al. (38), who examined the effect of dexamethasone on Na^+ transport in H441 cells, a bronchiolar epithelial cell line, as well as in A549 cells. Similar to the observations in A6 cells by Alvarez de la Rosa et al. (4), Itani et al. (38) noted an early and coordinate increase in all ENaC subunit mRNA levels in response to steroids. An increase in α-ENaC protein expression was also measured, but protein levels of the other subunits were not examined. This group also reported no change in the single-channel properties of ENaC channels in H441 cells in response to dexamethasone (38).

Na^+ reabsorption in alveolar type II cells is also regulated by β-adrenergic stimulation, and using electrophysiological methods, β-adrenergic agonists had previously been demonstrated to increase the number of active, highly selective cation channels in these cells (17). Recently, Planès and colleagues (67) examined the effect of β-adrenergic agonists on channel activity and apical membrane ENaC expression in normal and hypoxic alveolar type II cells. In both normal and hypoxic cells, apical α-ENaC represented 20–25% of the total cellular pool, comparable to our observation in A6 (81), whereas the apical expression of β- and γ-ENaC represented only ~5% of the total cellular pools. Amiloride-sensitive Na^+ transport was inhibited by ~45% in hypoxic cells; however, biochemically, this treatment caused a dramatic decrease (~75%) in the amounts of apical membrane β- and γ-ENaC with only a modest reduction (~30%) in cell surface α-ENaC (67). Stimulation of transport in hypoxic cells by addition of the β-adrenergic agonist tertbutaline restored amiloride-sensitive Na^+ transport to normal levels and caused a marked increase in apical β- and γ-ENaC expression without a change in apical α-ENaC (67). The effect of tertbutaline on short-ciruit current was blocked by brefeldin A, suggesting that membrane insertion of intracellular ENaC subunits was required for restoration of Na^+ transport (67). The authors interpreted their results to suggest that the trafficking of β- and γ-ENaC was highly sensitive to hypoxia and that decreased abundance of these two subunits would limit highly selective Na^+ channels. Consistent with this is the previous observation that the level of oxygenation influenced the expression of nonselective relative to highly selective Na^+ channels in alveolar type II cells (39).

FUNCTIONAL RELEVANCE OF NONCOORDINATE REGULATION

How do noncoordinate changes in individual ENaC subunit message and protein levels contribute to functional changes in Na^+ transport? One possibility is that noncoordinate increases in whole cell or apical membrane subunit levels simply represent excess subunits that have somehow escaped ER quality control and that are physiologically silent. Changes in the biochemical pools of subunits in whole cells or at the apical membrane do not necessarily mean changes in functional channels at the cell surface. On the other hand, the general concordance between changes in individual ENaC subunit levels and the rate of Na^+ transport suggests the possibility that changes in individual subunit expression can modulate cell surface ENaC properties (Tables 1 and 2). If noncoordinate regulation of ENaC is a physiologically relevant phenomenon, a minimal requirement is that either distinct single subunits or channels of diverse stoichiometry should exist in vivo under at least some physiological conditions. A further, more provocative implication is that fully active, preassembled heterotrimeric channels may recombine at post-ER sites into channels with different stoichiometries (and consequently different activities). Similar scenarios have previously been described for other heteromeric membrane proteins, which will be discussed below.

The notion of channels made of alternate stoichiometries is, by itself, not revolutionary. Subsets of ENaC subunits expressed in oocytes are known to form channels with varying subunit composition and transport properties (30, 58). In addition, a putative δ-subunit of ENaC has been cloned and shown to form functional Na^+ channels when coexpressed with β- and γ-ENaC (79). Similarly, H^+-gated cation channels, which have the same membrane topology as ENaC subunits, have the ability to coassemble to form channels with widely distinct properties (64, 80). Moreover, there are a variety of other heteromeric channels that also exhibit some tissue-specific plasticity in their ability to generate different channel properties, including some K^+ channels, P2X receptors, ligand-gated receptors, cAMP-gated channels, and GABA receptors (6, 16, 37, 62). Indeed, diversity in the ER export and post-ER trafficking motifs of G protein-activated K^+ channel subunits has recently been demonstrated to result in dramatically different trafficking itineraries of distinct heterotetrameric channel combinations (37, 52).

While the notion of postsynthetic remodeling of heteromeric complex stoichiometry is somewhat heretical, there are several instances in which assembly and disassembly of multimeric protein complexes have been proposed to occur at post-ER sites. For example, several dimeric apical membrane hydrolases, including dipeptidylpeptidase IV and lactase-phlorozin hydrolase, have been demonstrated to leave the ER as monomers and oligomerize only on reaching the Golgi complex or cell surface (19, 40). Similarly, some connexins (constituents of gap junctions) do not assemble into hexamers until they reach post-ER compartments (60, 82). Recently, the 17-kDa proteolipid subunits that comprise the V_0 pore-forming subunit of the H^+-ATPase were suggested to catalyze membrane fusion by forming head-to-head associations and then laterally disassembling (presumably in a reversible fashion), thereby allowing lipids to invade the pore region (66).

Perhaps the most compelling example of a heteromeric protein whose subunits can be trafficked in a noncoordinate fashion is the T cell antigen receptor-CD3 complex (TCR-CD3). This complex has long been considered a model system for the assembly and trafficking of multimeric membrane proteins (43). On the basis of studies primarily carried out in T cell hybridomas, a model for trafficking of this heptameric receptor was developed in which many of the components may by synthesized in excess and degraded (as has been observed with ENaC) but only combined into mature TCR-CD3 complexes and trafficked to the cell membrane when the putatively limiting ζ-subunit is synthesized (43, 59). This widely accepted model does not, however, fully explain the regulation of TCR-CD3 expression and trafficking in normal T cells, where significant differences were noted compared with the assembly and transport observed in T cell hybridomas (2, 65). In an elegant study by Ono et al. (65), the ζ chain of TCR-CD3 complexes at the cell surface was found to be degraded much more rapidly than the other cell surface subunits. Moreover, newly synthesized ζ-subunits were able to replace the degraded chains in cell surface TCR-CD3 complexes (65). Additionally, the other subunits that comprise cell surface TCRs are also apparently degraded at different rates, and it has been proposed that longer lived subunits may recycle and combine with newly synthesized chains to reestablish TCR-CD3 membrane receptors (2).

Distinct fates of individual chains of multimeric membrane proteins have also been noted for the α-, β-, and γ-chains of the interleukin 2 receptor (IL2R) in T cells. When this receptor is expressed at the surface of T cells, the α-subunit has a relatively long half-life at the plasma membrane, whereas the half-lives of cell surface IL2R β- and γ-subunits are quite short (~1 h) (34, 35). Differences in half-life of these subunits appear to be related to differing endocytic fates: the α-subunit of the IL2R is internalized into transferrin-positive, presumably recycling, compartments, whereas the β- and γ-subunits are largely excluded from these endosomes and do not colocalize with α.

Three nonexclusive models that describe a functional role for noncoordinate expression of ENaC subunits in cells and at the apical membrane are shown in Fig. 1. All of the models assume that the three ENaC subunits are synthesized in the ER at different rates, depending on tissue and hormonal stimuli. In the simplest model (*model A*), newly synthesized subunits accumulate in the ER or later compartments. When all three subunits are present, they combine into heterotetrameric channels and traffic to the apical membrane. This model is consistent with the observation that aldosterone stimulation of α-ENaC synthesis re-

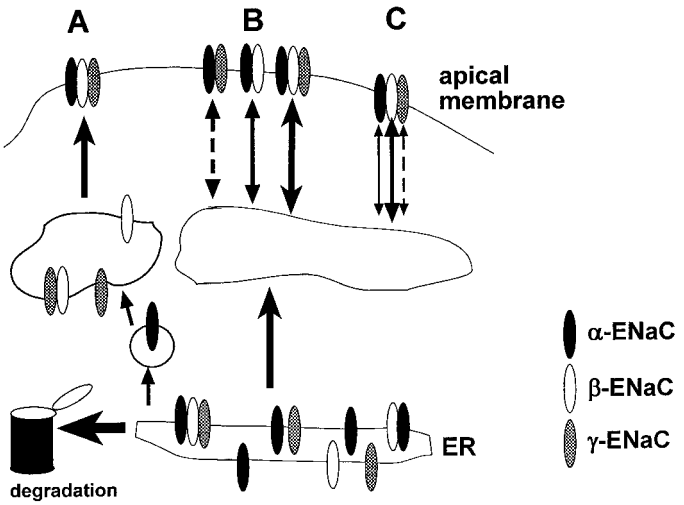

Fig. 1. The 3 models (*A–C*) described in the text for noncoordinate regulation of ENaC subunit assembly and trafficking are illustrated. Combinations of ENaC subunits (αβγ, αβ, and αγ) are drawn schematically and are not intended to depict the true stoichiometry of these complexes but simply to indicate the constituent subunits.

sults in movement of cytosolic β- and γ-ENaC to the apical membrane (53) but does not explain the changes in cell surface densities of individual ENaC subunits that parallel alterations in transport rates (67, 81). In *model B*, channels of varying stoichiometry are assembled in the ER and traffic to and from the apical membrane with distinct kinetics. ENaC trafficking in this model is analogous to that described for distinct combinations of K^+ channel subunits (52). For example, turnover of a population of apical homomeric α-ENaC channels and replacement by fully functional αβγ-channels would result in increased channel activity, with a selective increase in apical β- and γ-ENaC such as has been described in hypoxic alveolar type II cells in response to terbutaline (67). Finally, in *model C*, we consider the possibility that ENaC channels can reversibly disassemble and recombine at post-ER sites. Selective removal and internalization of individual subunits from channels at the apical membrane or in endosomal compartments, analogous to the dynamic remodeling proposed for the T cell receptor (2), could result in significant changes in cell surface ENaC activity without affecting the densities of other channel subunits. Conversely, delivery of individual subunits to the membrane might stimulate the recombination of existing cell surface subunits into channels with altered conductance. Remodeling of channels in response to up- or downregulation of subpopulations of ENaC subunits could potentially occur at any site along the biosynthetic pathway. While this model can explain all aspects of noncoordinate regulation, there are some troublesome ramifications. While *models A* and *B* are consistent, with assembly of tetrameric channels of a single or varying composition, *model C* predicts the existence (at least transiently) of residual, nontetrameric ENaC subunit assemblies generated by the selective removal of individual subunits from heterotetramers. The fate and channel activity of such nontraditional subunit assemblies are unclear as is the localization or precise nature of the cellular machinery capable of mediating such a process. Moreover, *model C* implies the presence of a steady-state pool of intracellular or recycling ENaC subunits that can rapidly be inserted in the membrane in response to physiological stimuli such as vasopressin.

Available evidence in epithelia that express endogenous ENaC do not presently allow us to distinguish among these models for ENaC trafficking. Data from rat kidney suggest there may be distinct intracellular pools of unassembled ENaC subunits, consistent with all three models (49, 53, 63). The differential trafficking or turnover of apical membrane ENaC subunits observed in experiments in cultured cells where channel function is physiologically regulated is consistent with *models B* and *C* (67, 81); however, these observations have recently been challenged by the report that surface ENaC subunits in these cells turn over with extremely rapid kinetics (4). A clear distinction between these latter two models will require a determination of apical membrane ENaC stoichiometry under various physiological conditions: *model B* predicts changes in the cell surface levels of complexes with fixed stoichiometries, whereas *model C* predicts a change in ENaC stoichiometry in response to certain physiological stimuli. In addition, identification of the subcellular compartments in which ENaC subunits reside, evidence for the recycling of ENaC subunits, and identification of signals that regulate apical targeting and internalization of individual subunits will be key to an understanding of the complex physiological regulation of ENaC function.

CONCLUDING REMARKS

Trafficking and regulation of the multisubunit channel complex ENaC have been studied and characterized extensively in oocytes and cellular expression systems. The complex is composed of three subunits that are synthesized in excess of apical membrane expression and are rapidly degraded. Subunits that assemble into heterotrimeric channels travel to the apical membrane, where they are short-lived and relatively rapidly degraded. In cells and tissues that express endogenous ENaC, the picture is somewhat more complex, and although this model is largely correct, there are also suggestions that apically expressed channel subunits may be longer lived and may be subject to recycling and post-ER recombination. Individual subunits of other heteromeric membrane proteins have been found to have different turnover rates at the plasma membrane in endogenously expressing cells, a phenomenon not initially recognized in studies using hybridomas or overexpression systems (2, 35, 65). The present data in tissues and cells that express the channel endogenously suggest the possibility that diversity in the trafficking and turnover of individual subunits may provide a new mechanism for the dynamic regulation of ENaC channel density in the apical membrane.

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DISCLOSURES

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