INVITED REVIEW

The epithelial sodium channel δ subunit: new notes for an old song

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Abstract

Amiloride-sensitive epithelial Na⁺ channels (ENaC) can be formed by different combinations of four homologous subunits, named α, β, γ and δ. In addition to providing an apical entry pathway for transepithelial Na⁺ reabsorption in tight epithelia such as the kidney distal tubule and collecting duct, ENaCs are also expressed in non-epithelial cells, where they may play different functional roles. The δ subunit of ENaC was originally identified in humans and is able to form amiloride-sensitive Na⁺ channels alone or in combination with β and γ, generally resembling the canonical kidney ENaC formed by α, β and γ. However, δ differs from α in its tissue distribution and channel properties. In spite of the low sequence conservation between α and δ (37% identity), their similar functional characteristics provide an excellent model to explore structural correlates of specific ENaC biophysical and pharmacological properties. Moreover, the study of cellular mechanisms modulating the activity of different ENaC subunit combinations provides an opportunity to gain insight into the regulation of the channel. In this review we examine the evolution of ENaC genes, channel subunit composition, the distinct functional and pharmacological features that δ confers to ENaC and how this can be exploited to better understand this ion channel. Finally, we briefly consider possible functional roles of the δ ENaC subunit.

Keywords: ENaC, ASIC, subunit composition, structure-function, pharmacology
Background information

The amiloride-sensitive epithelial Na\(^+\) channel (ENaC) is a multimeric ion channel formed by subunits belonging to the ENaC/degenerin ion channel family, which are genetically related glycoproteins with two transmembrane-spanning regions, intracellular NH\(_2\) and COOH-termini and a large extracellular domain (6, 49). ENaC can be formed by different combinations of four homologous subunits, named \(\alpha\), \(\beta\), \(\gamma\) and \(\delta\). A functional cloning approach in *Xenopus* oocytes using a rat colon cDNA library led to the isolation of three ENaC subunits (\(\alpha\), \(\beta\) and \(\gamma\)), each encoded by a different gene (18, 19). Co-expression of \(\alpha\), \(\beta\) and \(\gamma\) forms functional channels with properties indistinguishable from that of the native channels in principal cells of the collecting duct (19), which provide an apical Na\(^+\) entry pathway for Na\(^+\) reabsorption as a fine-tuning mechanism to control Na\(^+\) homeostasis and thus extracellular volume and blood pressure (6, 49). ENaC has also been shown to be essential for lung alveolar fluid homeostasis (3, 26), although the subunit composition of lung amiloride-sensitive Na\(^+\) channels displays far more complexity than in the kidney (see below). Soon after the cloning of \(\alpha\), \(\beta\) and \(\gamma\), a fourth subunit was reported in human tissues (87). This subunit, named \(\delta\), is most similar to \(\alpha\) in amino acid sequence (37% identity) and is able to form channels alone or in combination with \(\beta\) and \(\gamma\). The four ENaC subunits have been shown to be expressed in different combinations in non-epithelial tissues, where they may play diverse functional roles (49). Most importantly, \(\delta\) ENaC differs from \(\alpha\) in its tissue distribution and channel properties. It is highly expressed in tissues not directly related to Na\(^+\) reabsorption, including brain, although it is also present at low levels in the kidney and lung (87). Moreover, it is a constitutively active channel in heterologous expression systems, but its activity is potentiated by a decrease in extracellular pH (40, 97). Both the expression patter of \(\delta\) ENaC and its potentiation by extracellular protons make it similar to acid-sensing ion channel (ASIC) subunits, which are also members of the ENaC/degenerin family, highly expressed in nervous tissue and gated by protons (6, 49).
Low levels of expression of δ ENaC mRNA in human kidney (87) and the apparent absence of this gene in rats and mice initially diminished the interest towards this subunit. However, in recent years δ ENaC has been the focus of an increasing number of studies. In this review, we will focus on recent advances on the study of δ ENaC and how they provide a useful tool to better understand evolution, structure, function and regulation of canonical ENaC.

Evolution of ENaC subunit genes

Analysis of ENaC subunit genes using Ensembl (29) (www.ensembl.org, release 66 - February 2012) reveals that in general there exist four different ENaC subunit genes, the canonical α, β and γ genes, and an “α-like” gene, δ (named scnn1a, scnn1b, scnn1g and scnn1d respectively). These four genes are common to tetrapods as well as the coelacanth (Latimeria chalumnae), and are therefore common to Sarcopterygians (Fig. 1A). Annotation of the sea lamprey (Petromyzon marinus) genome reveals the presence of α, β and γ genes, but not of δ. The low-pass sequencing of the lamprey genome precludes any conclusion about the presence of a δ gene. In any case, the data place the origin of ENaC subunit genes before the sarcopterygian lineage gave rise to lobe-finned fishes and tetrapods (50), prior to the vertebrate water-to-land transition, and it is generally consistent with the appearance of eight genes in the ENaC/degenerin family of ion channels (6) by two rounds of genome duplication at the root of the vertebrate lineage (83). A search for new ENaC subunits in Xenopus laevis yielded an “α-like” subunit that was almost equally distant to Xenopus α and human δ (42% and 36% identity, respectively), and therefore was named ε (10). However, the ε Xenopus gene has been annotated as δ (gene name: scnn1d) by the National Center for Biotechnology Information (NCBI). Furthermore, upon comparison of the coding sequences available in Ensembl it is apparent that Xenopus tropicalis has a fourth ENaC gene in addition to scnn1a, scnn1b and scnn1g, which clusters with the δ subgroup of sequences (Fig. 1B). Remarkably, there are two independent X. tropicalis genes clustering with the δ group, suggesting a duplication
event of scnn1d in that organism (Fig. 1B). *Xenopus laevis* also has two additional \( \beta \) and \( \gamma \) subunit genes, named \( \beta_2 \) and \( \gamma_2 \), a fact that has been attributed to this organism’s polyploidy (10, 70).

The presence of a functional \( \delta \) ENaC gene in rodents has been controversial and requires closer examination. The gene is present in guinea pigs (*Cavia porcellus*), but not in mouse or rat (Ensembl, release 66, February 2012) unlike scnn1a, which is present in all three species (compare Fig. 1B and Fig. 1C). Therefore some rodents have the gene, but apparently it is not present in rats and mice.

It has been generally assumed that rat or mouse lack \( \delta \) ENaC (14). Indeed, as of February 2012, there is no record for rat scnn1d at the NCBI (www.ncbi.nlm.nih.gov/gene). The *Mus musculus* record lists it as a pseudo-gene based purely on *in silico* predictions. Briefly, this is supported by three observations: 1) When the mouse genome is searched using as query sequence the human delta ENaC gene only a partial hit is found in chromosome 4. The position of neighboring genes is consistent with the location of scnn1d in the syntenic region of human chromosome 1 (Fig. 2A); 2) A model with the mouse genome hit predicts a protein with only 48% similarity to amino acids 374-472 of the human protein (for comparison, human and mouse \( \alpha \) share 82% identity); 3) The mouse EST database found no transcript support for the expression of this sequence. On the other hand, there is one report in the literature that finds evidence for \( \delta \) transcript and protein in mouse sperm (34). While it may be arguable that the RT-PCR result represents a transcript from the pseudo-gene, the band shown in the western blot of this study is more difficult to explain and may be a result of nonspecific binding of the antibody.

To shed light on this issue, we constructed phylogenetic trees between ENaC subunit mRNAs for the eight species for which sequences for all four scnn genes were publicly available (Fig. 2B). Since no mRNA sequence for *Mus musculus* \( \delta \) was available for phylogenetic analysis, only conserved genomic regions were used, filtering out translated sequences with <50% conservation with other ENaC genes. Even using the least genetically differentiated segments of the gene, the
genetic distance between $\delta$ from *M. musculus* and the other species is almost 3 times the distance obtained for $\alpha$, $\beta$ and $\gamma$ (Fig. 2B). This evidence strongly suggests that *M. musculus* $\delta$ ENaC gene has evolved under less genetic pressure than its counterparts, consistent with the idea that the mouse *scnn1d* constitutes a pseudo-gene, which in turn would explain the difficulties of finding expression of $\delta$ ENaC in this species. Also, it is important to highlight that so far there are no reports of full-length cloning of $\delta$ ENaC cDNA from a species different from humans (excluding the *Xenopus* $\varepsilon$ subunit). There are reports of a partial mRNA sequence detected by PCR in rabbit retina (15), although no sequence support for rabbit $\delta$ ENaC is available from NCBI or Ensembl. Supporting evidence for $\delta$ transcripts are now available for chimpanzee and cows (Ensembl), as well as for *Macaca mulatta* (32). Until more data is available, and taking into consideration the information gathered from mice, we cannot be certain that $\delta$ ENaC is a transcribed gene in every species where the sequence (or parts of it) is found.

$\delta$ ENaC expression and combination with other subunits

The pattern of $\delta$ ENaC expression differs from that of $\alpha$ and does not correlate well with a physiological role in aldosterone-targeted Na$^+$ transporting epithelia. The original description of human $\delta$ found prominent expression in human gonads, pancreas, brain, heart and thymus, with lower amounts in aldosterone-targeted kidney and lung (87). Later, it was found by dot-blot studies that the expression of $\delta$ is widespread, including heart, liver, pancreas, different areas of the brain, stomach/esophagus, skeletal muscle and, interestingly, both adult and fetal human kidney (97). We could not detect the expression of $\delta$ in kidney or lung by RT-PCR, but we confirmed prominent expression in pancreas, brain and skeletal muscle (32). Lung alveolar epithelial cell lines such as H441 cells express $\delta$ ENaC (42, 63) and there are reports showing the presence of $\delta$ in lung tissue (3, 63, 101). The presence of $\delta$ has also been reported in the epithelium lining the human esophagus, where it is co-expressed with $\alpha$, $\beta$ and $\gamma$ (92). Within the central nervous system, we have
demonstrated that in the human and monkey brain $\delta$ is restricted to neurons (32). Two isoforms of the protein with distinct N-terminal regions have been reported (32, 96). Both $\delta$ isoforms are expressed in pyramidal cells of the human and monkey cerebral cortex and in different neuronal populations of telencephalic subcortical nuclei, but double-labeling experiments demonstrated a low level of co-localization between isoforms (5-9%), suggesting specific functional roles for each of them (32, 88). No detectable expression has been found in glial cells in human and monkey brain samples (32), although cultured glioblastoma cells do express $\delta$ (13), suggesting that under certain pathological situations $\delta$ expression patterns may vary considerably.

The situation becomes even more complex when the other subunits are considered. Both $\delta$ and $\alpha$ form channels alone, but their combination with $\beta$ and/or $\gamma$ subunits increase their functional expression by more than one order of magnitude (19, 87). At least in human lung and esophagus, it is conceivable that the four subunits are co-expressed in the same cells (3, 92). In other tissues, different combinations are possible. For instance, pyramidal neurons of the human brain cortex prominently express $\delta$, but not other ENaC subunits (32). It has been proposed that physical interaction of $\delta$ with $\alpha\beta\gamma$ channels modifies ENaC properties (42, 63) and that such interactions may underlie the detection of native channels with properties different from the canonical ENaC in lung alveolar cells (3, 63) or in glioma cells (45). Given the presence of $\delta$ in cells where no $\alpha$, $\beta$ or $\gamma$ is expressed, together with the fact that in heterologous expression systems homomeric $\delta$ channels produce very low current levels when compared to heteromers (32, 87) due to low plasma membrane expression (32), it is tempting to speculate that in the nervous system $\delta$ may interact with other proteins from the ENaC/DEG family (6), such as ASIC subunits. Indeed there is experimental data supporting the existence of hybrid ENaC/ASIC channels in heterologous expression systems (46, 62). The possibility of ENaC channels formed by different subunit combinations brings forward the question of ENaC stoichiometry.
The stoichiometry of ENaC has been a matter of long debate. The most studied case is the co-expression of α, β and γ subunits. A combination of biochemical, electrophysiological and fluorescence techniques have provided evidence supporting either a tetrameric structure with two α, one β and one γ subunit (8, 28), or alternatively a higher order heteromer formed by up to nine subunits (77, 79, 81). The publication of a resolved crystal structure of ASIC1 showing that it associates as a trimer (38) has led to a proposal of a trimeric structure for αβγ ENaC (17, 82). Even though there is poor sequence conservation between ASIC1 and ENaC subunits, there is evidence supporting structural homology between these proteins, at least in the extracellular domains (47). This observation of structural homology by molecular modeling and the common evolutionary origin of ASIC and ENaC supports a heterotrimeric structure for the channel. However, there still remain some observations that are not accounted by this fact, specially the modification of αβγ properties by co-expression of δ subunits (42, 63; see below). So far there have been no studies directly studying the stoichiometry of δ-containing ENaC channels. Much remains to be explored about other partners that may interact with δ ENaC and the stoichiometry of such associations.

What can δ teach us about ENaC function?

Heterologously expressed δβγ channels show several distinctive features differing from αβγ channels (summarized in Table 1). The first interesting aspect is the conduction properties. The unitary conductance of δβγ channels is higher than that of αβγ (12 pS vs 6 pS, respectively) (19, 87). Similarly to αβγ, the channels formed by δβγ show higher selectivity for Na⁺ over K⁺. Nevertheless, δβγ channels are more selective to Na⁺ over Li⁺ ($I_{Li^+}/I_{Na^+} = 0.6$), as opposed to αβγ channels, which have the reverse relationship ($I_{Li^+}/I_{Na^+} = 2$) (19, 87). Ion selectivity properties in α and δ homomeric channels show the same characteristics as their respective trimer. The differences in conduction allowed Ji et al. (41) to use chimeric constructs based on the swapping of transmembrane domain 2 (TM2) and adjacent areas between α and δ. The authors concluded that an interaction between the hydrophobic domain preceding TM2 (H2) and the amino acids just prior to H2 (pre-H2) participate
in setting ion selectivity and single channel conductance properties (41). Another interesting feature
of δβγ channels is that they have a significantly higher open probability (Po) than αβγ channels (33, 88). The structural basis for this difference is unknown, although it may have to do with the
differential regulation of both types of channel by extracellular proteases (33) (discussed below).

It has been shown that the δ subunit confers sensitivity to extracellular protons, with a half-maximal
pH for activation in the range of 5 to 6 (40, 97). The effect of pH on ENaC channels is strongly
dependent on subunit composition and species. Thus, whereas rat αβγ ENaC expressed in Xenopus
oocytes is not affected by external pH variations (40, 100), human αβγ currents are enhanced by
protons, a mechanism that may facilitate Na⁺ reabsorption under acidosis (23). On the other hand,
channels formed by rat α and β subunits ENaC are inhibited by acidic pH (100). The progressive
and sustained increase in activity shown by δ in response to extracellular protons makes it an ideal
pH sensor. It is not known which precise residues are involved in δ subunit proton sensitivity.

Since human αβγ channels are also activated by a drop in extracellular pH, it could be possible that
the sensitivity to protons is given by β and γ. This last possibility seems unlikely because
homomeric δ channels are also activated by extracellular pH (97).

The proton sensitivity brings an intriguing (and still unanswered) question: are δ containing
channels part of the proton activated Na⁺ channels in neurons or other cell types? (53) The answer
to this question is still not clear. As mentioned above, the δ subunit is expressed in several types of
neurons where different ASIC subunits are present (32) and this situation may also be true in other
cell types. In heterologous systems it has been shown that ASIC1 and ASIC2 can form heteromers
with all ENaC channel subunits (46). It is then possible that this interaction occurs in vivo; however
the evidence is not conclusive and the lack of endogenous δ subunit expression in mouse and rat has
been a pitfall to solve this question.
A distinctive feature of ENaC channels is the decrease in current amplitude due to external Na\(^+\), a phenomenon called sodium self-inhibition (31). This is evident as a transient increase and subsequent decrease in Na\(^+\) current after amiloride washout. Several studies have identified extracellular domain regions and residues involved in the allosteric inhibition of αβγ channels by Na\(^+\). Several positively and negatively charged residues in the α and γ subunit (particularly γ H235), as well as cysteines involved in forming intrasubunit disulfide bonds play a role in the effect (27, 60, 74, 75, 90). The ε subunit cloned from *Xenopus laevis*, which as stated above probably represents an ortholog of the δ subunit, shows slower Na\(^+\) self-inhibition when co-expressed with β and γ than *Xenopus αβγ* channels; however, the percentage of current inhibited is much larger (10). To identify regions involved in this differential behavior, the authors constructed a set of chimeras swapping different regions of ε and α subunits, which clearly showed that the crucial region for determining Na\(^+\) self-inhibition is located in the extracellular domain of the subunits (10). Na\(^+\) self-inhibition has also been compared between human αβγ and δβγ channels expressed in *Xenopus* oocytes. Consistent with the results found with *Xenopus ε*, δβγ channels showed a slower self-inhibition than αβγ (42). Interestingly, it has been demonstrated that protons increase current by relieving Na\(^+\) self-inhibition at human αβγ channels (23). If this were the case for δ channels it would generalize the model where proton binding alters extracellular regulation of ENaC by Na\(^+\).

Rapid regulation of ENaC activity by changes in extracellular ion composition and pH, combined with the associated structure-function analysis, suggests that the extracellular domain of the channel serves as a sensor to modulate channel activity in response to changes in the environment. This idea is reinforced by the fact that ENaC is regulated by changes in extracellular fluid flow (71), which may in turn alter hydrostatic pressure, membrane stretch, and shear stress. It was later demonstrated that laminar shear stress regulates the open probability of αβγ ENaC expressed in *Xenopus* oocytes as well as native channels in epithelial cells (2, 20). The magnitude and time course of the response of αβγ and δβγ channels to laminar shear stress are notably different (1). The magnitude of the
response may reflect differences in open probability (Po) between both types of channels, since $\delta \beta \gamma$
has a much higher Po than $\alpha \beta \gamma$ (33, 88). On the other hand, the use of chimeras between $\alpha$ and $\delta$
subunits allowed the authors to conclude that residues in TM2 and H2 were involved in determining
the time course of the response (1), which probably reflects the coupling between stimulus sensing
and the modification of gating. The actual extracellular domain structure involved in sensing flow is
still unknown. ENaC sensitivity to shear force may also explain the functional role of this channel
when expressed in other tissues exposed to flow such as the vascular endothelium or in touch-
sensitive structures such as the rat foot pad (30).

How does $\delta$ENaC pharmacology differ from canonical ENaC channels?

Another distinctive feature of $\delta$ and $\delta \beta \gamma$ channels compared to $\alpha$ or $\alpha \beta \gamma$ is a 30-fold decrease in the
sensitivity for amiloride and its analog benzamil (87), two well-known blockers of ENaC with
clinical use as potassium-conserving diuretics (56). Furthermore, amiloride binding to $\delta \beta \gamma$ shows
higher voltage-dependence than in the case of $\alpha \beta \gamma$ (41). Other subunit combinations such as $\alpha \beta$ and
$\alpha \gamma$ also showed altered amiloride and benzamil apparent affinities (61). Amiloride occludes the pore
from the external side of the membrane (49), but in the absence of a high-resolution crystal
structure of ENaC, the precise amino acid residues involved in amiloride binding are still undefined.
The use of site-directed mutagenesis to study amiloride binding determinants is limited by the fact
that the same mutants that alter amiloride affinity also affect channel conductance and permeability
properties (49). However, the currently accepted model involves the binding of the guanidium
moiety of amiloride to the outer mouth of the selectivity filter (76), with the pyrazine ring binding
to an area preceding TM2 (48, 52, 72, 76). These observations were fundamental to demonstrate
that all subunits line the ENaC pore (49), which is consistent with the observations showing the
influence of ENaC subunit composition in amiloride binding. The use of chimeric subunits
swapping H2 between $\alpha$ and $\delta$ further corroborated the role of this region in determining amiloride binding to ENaC (41).

Recently, a small molecule activator of ENaC was described (59). Compound S3969 increases ENaC open probability only in heteromeric channels that incorporate either $\alpha$ or $\delta$ together with $\beta$ and $\gamma$ subunits. The compound is equally effective in $\alpha\beta\gamma$ and $\delta\beta\gamma$ channels (59). Activation of ENaC by S3969 critically depends on residue V348 in the extracellular domain of the $\beta$ subunit, but it needs the presence of $\gamma$ and $\alpha$, which in turn can be substituted by $\delta$. The low sequence identity between $\alpha$ and $\delta$, together with the fact that both subunits can be exchanged without altering S3969 effect, suggests that the binding site is formed by $\beta$ and $\gamma$ and that $\alpha$ and $\delta$ participate in a conserved mechanism that transduce the effect to the pore. These findings indirectly support the idea that ENaC, like other members of the ENaC/DEG family, may be a ligand-gated ion channel (6, 59).

In the past few years various pharmacological agents have been proposed as selective modulators of $\delta$ ENaC. Capsazepine, previously known as a competitive antagonist of TRPV1 activation by capsaicin (65), was reported to be an activator of $\delta\beta\gamma$ ENaC (93). Application of other vanilloid compounds such as capsaicin, resiniferatoxin, and olvanil, or a structurally related compound (dopamine) did not modulate $\delta\beta\gamma$ activity. Although $\delta$ ENaC homomeric channels were also significantly activated by capsazepine, the drug had no effect on human $\alpha$ ENaC, while it produced a slight decrease in the $\alpha\beta\gamma$ current. Another $\delta$-specific activator has been described, icilin (95). Both homomeric $\delta$ channels and $\delta\beta\gamma$ were activated by icilin, whereas $\alpha$ activity was not affected and $\alpha\beta\gamma$ was slightly inhibited (95). Finally, a well-know dye, Evan’s blue, was reported to display specific $\delta$ antagonist activity (98), with no effect on $\alpha$ subunits. Unfortunately, these three reagents have multiple other targets in the cell, including $K^+$ ion channels (91), which in turn can modify transepithelial $Na^+$ transport (4). Therefore, their use to specifically dissect the contribution of $\delta$ subunit to ENaC currents in cells and native tissues have been limited (73).
Differential effect of subunit composition on cellular control of ENaC abundance and activity in the plasma membrane

Tight control of ENaC abundance and activity in the membrane is essential to modulate transepithelial Na\(^+\) reabsorption. In addition, ENaC expression in non-epithelial cells may lead to an excessive Na\(^+\) leak that would be deleterious for the cell. Therefore, it is not surprising that elaborate machinery has evolved to control ENaC trafficking to and from the membrane (16). The steroid hormone aldosterone, the main regulator of ENaC activity, redistributes channels to the apical membrane in principal cells of the distal kidney tubule, at least in part by decreasing ENaC endocytosis rate (57). ENaC stability in the membrane depends at least in part on the activity of the ubiquitin ligase Nedd4–2, which interacts with ENaC subunits through a COOH-terminal PY motif (PPxY) (44, 80). This process is controlled by the serum- and glucocorticoid-induced kinase 1 (SGK1), a serine/threonine kinase transcriptionally regulated by aldosterone (54). SGK1 is a known activator of ENaC, mainly by enhancing steady-state ENaC abundance in the plasma membrane (7). SGK1 phosphorylates Nedd4–2 and disrupts its interaction with ENaC, stabilizing the channel in the membrane (25, 78). Recently, SGK1.1, a new splice isoform of SGK1 with prominent expression in the central nervous system, has been described (9). SGK1.1 has a different NH\(_2\)-terminal domain that confers enhanced stability and binding of SGK1.1 to the plasma membrane by interaction with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P\(_2\)). SGK1.1 regulates the activity of ASIC1 (9), a member of the ENaC/DEG family and is co-expressed with \(\delta\) ENaC in pyramidal neurons of the human and monkey cerebral cortex (89). We have recently shown that SGK1.1 regulates homomeric ENaC \(\delta\) as well as \(\delta\beta\gamma\) channels (89). Interestingly, \(\delta\) lacks a PY motif in its COOH-terminal domain, indicating that SGK1.1 and possibly Nedd4-2, can regulate ENaC channels independently of PY motifs. In addition, our results showed that physiological or pharmacological activation of phospholipase C, which decreases PtdIns(4,5)P\(_2\) abundance, abrogates SGK1.1 interaction with the plasma membrane and modulation of \(\delta\) ENaC. SGK1
isoform activation depends on the phosphoinositide-3 kinase pathway (66). Therefore, SGK1 isoforms probably provide a convergence point of different signaling pathways regulating ENaC in a cell-type specific mode (5, 67, 89).

Regulation of ENaC delivery to the plasma membrane is less well studied. It is clear that there are constitutive and hormonal-regulated ENaC traffic pathways to the plasma membrane and the involvement of small GTPases and other factors have been established (16). However, little is known about sequence determinants in ENaC subunits involved in trafficking of newly synthesized or recycled channels to the membrane. We have recently taken advantage of the existence of the two splice isoforms of human δ ENaC with divergent NH2-terminal sequences (32, 96) to study whether there are trafficking differences between them. Our results showed that δ1βγ and δ2βγ expression in *Xenopus* oocytes produces different current densities that can be fully explained by differential trafficking of the channels (88). Since the difference is also apparent in the absence of β and γ, the underlying mechanism is independent of PY motifs. Serial deletion of δ2 NH2-terminal domain identified two independent amino acid regions responsible for the differential membrane abundance, which most likely reflect distinct channel insertion rates in the membrane (88). Finally, dynasore, a blocker of dynamin-independent endocytosis, increased αβγ channel abundance in the membrane, but failed to modulate δβγ (88). This suggests that δ-containing channels may follow an alternative endocytosis rate, which in turn would indicate that δ is dominant over β and γ on setting at least some trafficking properties of ENaC.

The fact that some aspects of δ trafficking such as SGK1.1-regulated increase in channel membrane abundance or differential δ isoform insertion rates are independent of PY motifs do not imply that δ endocytosis is not regulated by ubiquitination. In fact, it has been demonstrated that COMMD1 (copper metabolism Murr1 domain 1) associates with δ ENaC and downregulates its activity by decreasing channel membrane abundance, while simultaneously increasing δ ubiquitination (14, 22).
The internalized channels are sorted to the early/recycling endosomal pool, suggesting a new pathway for regulation of δ ENaC.

One additional cellular mechanism controlling ENaC activity in the membrane is the increase in channel Po by proteolytic processing of the extracellular domain, a mechanism that has been extensively studied in αβγ channels (51). Recently, Haerteis et al. examined whether δβγ channels undergo proteolytic processing as well, uncovering another functional difference triggered by the substitution of α by δ in ENaC heteromers (33). Basal δβγ ENaC Po was found to be high (approx. 0.9) and significantly larger than αβγ Po, an observation later corroborated by our group (88).

Consistent with the larger Po in δβγ channels, current stimulation by treatment with chymotrypsin was smaller in δβγ than in αβγ (33). Taken together, the results suggest that δ alters proteolytic processing of ENaC, increasing the relative size of a pool of channels with high Po. The authors also found that αβγ and δβγ plasma membrane abundance is similar, although δβγ channels produced a much larger whole-cell current (a combined effect of larger conductance and higher Po).

In our hands, αβγ channels consistently produce larger whole-cell current, which is explained by lower plasma membrane abundance of both δ1βγ and δ2βγ [(32) and our unpublished observations]. The reasons for this discrepancy are unknown.

**Possible physiological roles for δ ENaC**

Presently there is no report of Na⁺ transport activity mediated by δ containing channels in native cells, a fact that can be partially attributed to the difficulty of finding a good model. As mentioned above, there has been no report of functional δ expression in mouse or rat cells. The only direct attempt to identify the contribution of δ ENaC has been made in glioblastoma cells, which display an amiloride-sensitive cation conductance thought to be composed of a hybrid channel made of ENaC and ASIC subunits (45). The authors used a previously reported strategy that overexpresses truncated versions of ENaC/ASIC subunits to knock down protein expression levels through an
unknown mechanism (39). The results showed that knocking down ASIC1, α or γ partially
attenuated the cation conductance, while knocking-down δ did not have an effect on whole-cell
current. Preliminary attempts to detect the contribution of δ ENaC to transepithelial Na+ transport in
alveolar (A549, H441) and bronchial (Calu-3, 16HBE14o-) epithelial cell lines based on differential
modulation by pharmacological agents (see above) provided inconclusive results (73).

Still, there are several possible physiological roles for channels containing δ subunit. In several
neuronal types a persistent Na+ current (insensitive to tetrodotoxin) is responsible for repetitive
firing by causing a small but continuous Na+ influx enough to maintain a depolarization that will
reach the threshold (12). Several different channels have been suggested to be responsible of this
current, including NaV1.2 and NaV1.6 (12). Another candidate is the unconventional NALCN
sodium channel, which belongs to the family of NaV channels but lacks positive charges in the S4
segment, part of the voltage sensor. NALCN is responsible for part of the leak Na+ conductance in
brain stem (58). In this context, δ-containing ENaC channels are plausible candidates to mediate a
leak Na+ conductance due to their localization in primate neurons (32). It has been proposed that
NALCN serves a similar role in pancreatic β-cell physiology (85). Human pancreas is one of the
sites with high levels of δ ENaC mRNA expression (32, 87, 97). The site of expression of δ ENaC
in the human pancreas is unknown. To date, no amiloride-sensitive Na+ conductance has been
detected in pancreatic duct cells of different species (24, 64, 102). It remains to be seen if δ ENaC is
expressed in the endocrine pancreas, where it may play a role in controlling cell excitability. On
possibility to address these issues would be to express δ ENaC, alone or in combination with other
ENaC/DEG subunits, in primary cultures of excitable cells such as neurons and test how membrane
potential and excitability are affected. Another possibility would be to develop transgenic mice
expressing δ ENaC in specific cell types.
The observation that $\delta$ ENaC currents are potently enhanced by a drop in extracellular pH in a sustained manner led to the hypothesis that they may be important factors in the transduction of ischemic and hypoxic signals in neurons (40). Under hypoxic insults, membrane depolarization and increased intracellular Na$^+$ and Cl$^-$ have been observed. It is conceivable that $\delta$ ENaC channels provide at least part of this Na$^+$ influx. Additionally, $\delta$ may mediate acid sensing in esophagus epithelium (92) and skin (94), and sour taste perception (35). Finally, it has been proposed that $\delta$ containing channels could participate as pH sensors in the response to aspiration of acid in the lung (101).

Alveolar fluid clearance is essential for appropriate lung gas exchange and depends on ENaC-mediated transepithelial Na$^+$ reabsorption (3). As described above, alveolar epithelial cell lines and human lung tissue express the four ENaC subunits, $\alpha$, $\delta$, $\beta$ and $\gamma$, potentially forming different combinations of heteromeric channels (3, 42, 63). Three different amiloride-sensitive conductances have been described in alveolar epithelial cells based on their selectivity towards cations (26, 31): highly-selective Na$^+$ channels (HSC), which have a large selectivity for Na$^+$ over K$^+$, channels displaying moderate selectivity and non-selective cation channels (NSC), with no selectivity of Na$^+$ over K$^+$. HSCs are almost identical to $\alpha/\beta/\gamma$ channels expressed in Xenopus oocytes (36, 37). In contrast, the molecular identity of the two other groups has remained controversial. Work by Dr. Eaton’s group has led to the idea that NSC represent homomeric channels formed by $\alpha$ ENaC (36, 37). Given that the four ENaC subunits are present in the human lung, it is conceivable that different combinations of subunits may produce ENaC channels with different properties. The possibility of ENaC and ASIC subunits forming hybrid channels (62) further opens new possibilities, since lung epithelial cells also express ASIC subunits (84). Dissecting these possibilities will require appropriate models of $\delta$ ENaC expression with the use of subunit-selective knockdown or the development of more specific pharmacological tools.
In mouse sperm, δ ENaC has been hypothesized to participate in the hyperpolarization process involved in the capacitation process (34), although the expression of functional δ subunits in mice still needs to be demonstrated and, as noted above, there are several lines of evidence that points towards scnn1d being a pseudogene in M. musculus.

Concluding remarks

ENaC channels have attracted much attention due to their wide pathophysiological implications in epithelial tissues, specially the kidney and lung. A great deal of effort has been invested in trying to understand ENaC molecular composition, the structural basis of its function, pharmacology and regulation by a wide variety of mechanisms. In the past few years the field has further expanded with the growing realization that ENaC channels may have important pathophysiological roles outside tight epithelia. Contributing to it, there has been renewed interested in studying the “fourth” ENaC subunit, δ, which had been largely neglected due to low levels of expression in kidney and higher expression in non-epithelial tissues such as brain, where a candidate current corresponding to δ ENaC was not immediately obvious. In addition, the apparent absence of a functional a δ ENaC gene in mice and rats makes it difficult to develop animal models and perform in vivo expression analysis. In this review we have summarized current knowledge about ENaC subunit diversity and putative functional roles. In addition to the interest in studying ENaC function outside epithelia, much is being gained by comparing properties of ENaC composed of different subunits, particularly those channels incorporating αβγ and δβγ.
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**Figure legends**

**Fig. 1.** A. phylogenetic tree showing the four ENaC subunit gene clusters found in animals. Dark grey represents collapsed subtrees of δ ENaC (SCNN1D) paralogs (γ, β and α; SCNN1G, SCNN1B and SCNN1A respectively). Light grey represents collapsed subtree of SCNN1D. Sarcopterygians include all tetrapods and lobe-finned fish. B, partially expanded SCNN1D tree. C, partially expanded SCNN1A tree. In all panels, light grey squares represent genome duplication nodes; black squares represent speciation nodes.

**Fig. 2. Evolutionary rates of ENaC genes.** A, regions of human chromosome 1 and mouse chromosome 4 with shared synteny, including the human δ subunit gene (scnn1d) and the mouse scnn1d pseudogene (adapted from the NCBI Gene resource, genomic context tool, gene IDs 6339 – H. sapiens- and 140501 – M. musculus-). B, ENaC subunit mRNAs sequences from eight species (Homo sapiens, Macaca mulatta, Pan troglodytes, Cavia porcellus, Mus musculus, Bos Taurus, Canis familiaris and Equus caballus) were obtained from Ensembl. Since the only available sequence for Mus musculus δ ENaC was genomic, we only kept for phylogenetic analyses the most conserved regions to other ENaC genes. To that end, translated amino acid Expasy BLAST searches using frame-shifted sequences were performed, assuming that gene conservation might improve at amino acid level, followed by an elimination of sequences that did not attain >50% conservation with other ENaC genes. The three conserved regions (reminiscent of pseudo-gene exons) were used for phylogenetic analysis after alignments using Clustal X (55). TREEFINDER (43) was utilized to derive consensus trees from 1000 replicates of maximum likelihood trees obtained under the best fitting model for nucleotide substitution estimated from the data. Branches supported by >50% replicate trees are indicated. Even using the least differentiated parts of the gene, the genetic distance between δ from M. musculus and the other species is almost 3 times the distance obtained for α, β and γ (note the different scale on each tree). This result strongly suggests that M. musculus
δ ENaC gene has evolved under less genetic pressure than its counterparts, consistent with the notion of it being a pseudogene.
Table 1. Summary of functional differences between channels formed by different combinations of ENaC and ASIC subunits expressed in heterologous expression systems*

<table>
<thead>
<tr>
<th>Channel</th>
<th>Conductance ($g_{Na^+}$, pS)</th>
<th>Po</th>
<th>Aminoride IC$_{50}$ (μM)</th>
<th>Ion selectivity ($I_{Li^+}/I_{Na^+}$)</th>
<th>pHe $^\dagger$ dependence (EC$_{50}$ for H$^+$)</th>
<th>Effect of chymotrypsin $^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha\beta\gamma$</td>
<td>6 (19)</td>
<td>0.14 (33)</td>
<td>0.1 (19)</td>
<td>2 (19)</td>
<td>$\S$</td>
<td>5-fold (33)</td>
</tr>
<tr>
<td>$\delta\beta\gamma$</td>
<td>12 (87)</td>
<td>0.7-0.9 (33, 88)</td>
<td>2.6 (87)</td>
<td>0.6 (87)</td>
<td>5-6 (40, 97)</td>
<td>2-fold (33)</td>
</tr>
<tr>
<td>ASIC1a</td>
<td>23 (99)</td>
<td>pH dependent</td>
<td>10 (86)</td>
<td>0.8 (86)</td>
<td>6.4 (86)</td>
<td>0.1-fold (68)</td>
</tr>
<tr>
<td>ASIC2a</td>
<td>22.5 (99)</td>
<td>pH dependent</td>
<td>28 (21)</td>
<td>1 (69)</td>
<td>4.3 (21)</td>
<td>no effect (68)</td>
</tr>
<tr>
<td>ASIC3</td>
<td>18.5 (99)</td>
<td>pH dependent</td>
<td>60-100 (11)</td>
<td>3.7 (11)</td>
<td>no effect (68)</td>
<td></td>
</tr>
</tbody>
</table>

* Only a restricted set of subunit combinations has been included. For more information on the variability of homomeric and heteromeric ASIC channels, see reference (49)

$^\dagger$ pH, extracellular pH

$^\ddagger$ Change over basal current

$\S$ The pHe dependence of $\alpha\beta\gamma$ channels is species-specific: rat $\alpha\beta\gamma$ is unaffected, human $\alpha\beta\gamma$ is activated (23, 40, 100)