The heterotetrameric architecture of the epithelial sodium channel (ENaC)

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The epithelial sodium channel (ENaC) is a key element for the maintenance of sodium balance and the regulation of blood pressure. Three homologous ENaC subunits (α, β and γ) assemble to form a highly Na⁺-selective channel. However, the subunit stoichiometry of ENaC has not yet been solved. Quantitative analysis of cell surface expression of ENaC α, β and γ subunits shows that they assemble according to a fixed stoichiometry, with α ENaC as the most abundant subunit. Functional assays based on differential sensitivities to channel blockers elicited by mutations tagging each α, β and γ subunit are consistent with a four subunit stoichiometry composed of two α, one β and one γ. Expression of concatameric cDNA constructs made of different combinations of ENaC subunits confirmed that the arrangement of the subunits around the channel pore consists of two α subunits separated by β and γ subunits.

Keywords: amiloride/channel pore/ENaC/epithelial sodium channel/subunit stoichiometry

Introduction

The epithelial sodium channel (ENaC) belongs to the recently identified DEG/ENaC channel gene superfamily (Canessa et al., 1994b) which can be divided into four different branches, (i) the ENaC channel genes involved in Na⁺ reabsorption in epithelia, (ii) the voltage-independent brain Na channel genes (BNAC1 and BNAC2) (Garcia-Anoveros et al., 1997; Waldmann et al., 1997), (iii) the degenerins (MEC-4, MEC-10 and DEG-1) of the nematode Caenorhabditis elegans that form part of a mechanotransducing complex for touch sensitivity (Tavernarakis and Driscoll, 1997), and (iv) the FMRFamide peptide-gated sodium channel (FaNaCh) found in the snail Helix aspera (Lingueglia et al., 1995). The pathophysiological importance of ENaC recently has been evidenced by the identification of ENaC gene mutations responsible for an autosomal dominant form of hypertension (Liddle syndrome) and for a salt-losing syndrome termed pseudo-hypoaldosteronism type-1 (Shimkets et al., 1994; Chang et al., 1996).

All the members of the DEG/ENaC gene superfamily share in common a structure predicting two hydrophobic membrane-spanning regions, intracellular N- and C-termini and a large extracellular loop with highly conserved cysteine residues (Canessa et al., 1994a; Renard et al., 1994; Snyder et al., 1994; Lai et al., 1996). ENaC is a multimeric channel made of homologous α, β and γ subunits surrounding the channel pore (Canessa et al., 1994b; Schild et al., 1997). The subunit stoichiometry of members of the DEG/ENaC gene superfamily has not yet been resolved. In the present work, we report independent evidence indicating that in Xenopus oocytes expressing the α, β and γ subunits, the ENaC channel is composed of two α, one β and one γ subunit surrounding the channel pore.

Results

Cell surface expression of ENaC α, β and γ subunits

Assessment of the α, β and γ subunit stoichiometry of the heteromultimeric ENaC protein was achieved in the Xenopus oocyte expression system by determination of the number of individual α, β and γ subunits that form the channel pore. This approach assumes that the stoichiometry of α, β and γ subunits is fixed when the three subunits are co-expressed. In the Xenopus oocyte expression system, the α β γ subunits are necessary to obtain maximal expression of ENaC at the cell surface, indicating that >95% of functional channel complexes are made of the α, β and γ subunits (Firsov et al., 1996). To exclude the possibility that ENaC subunits assemble indiscriminately, we have first tested whether an α, β or γ subunit can substitute for one of its two homologues to form a functional channel. Binding of an iodinated monoclonal antibody (mAb) against an epitope in the extracellular domain of ENaC subunits was used to quantify cell surface expression of ENaC subunits in intact oocytes (Firsov et al., 1996). Pairs of subunits were tagged with a FLAG epitope (either βγ, αγ or αβ) and co-expressed with increasing cRNA concentrations of the untagged subunits (Figure 1A–F) and co-expressed with increasing cRNA concentrations of the untagged subunits (Figure 1A–F). In individual oocytes, specific binding of [125I]anti-FLAG mAb to FLAG-tagged ENaC subunits at the cell surface is linearly correlated with ENaC activity as measured by the amiloride-sensitive Na⁺ current (IₐNa) (Figure 1A, C and E) (Firsov et al., 1996). This linear correlation allows us to normalize the binding signal (in fmol) per μA of IₐNa, in other words to normalize the number of tagged subunits per active channel expressed at the cell surface. In the case of indiscriminate aggregation of ENaC subunits, competition between untagged and tagged subunits will result in a decrease in the binding (fmol/μA). In Figure 1B, D and F, the number of FLAG-tagged subunits expressed at the cell surface as
ENaC subunit stoichiometry

determined by specific binding of $^{[125]}$I-anti-FLAG mAb normalized for the measured current $I_{Na}$ (fmol/$\mu$A) remains constant, despite injection of increasing concentrations of cRNA encoding the untagged subunit. Thus when the three subunits are co-expressed, $\alpha$, $\beta$, and $\gamma$ subunits are not interchangeable, supporting the hypothesis that assembly of the subunits occurs according to a fixed stoichiometry. This lack of interchangeability among ENaC subunits signifies that upon expression of the $\alpha$, $\beta$, and $\gamma$ subunits with similar concentrations of cRNA message, the formation of $\alpha\beta$ or $\alpha\gamma$ channel complexes is unlikely, and if present they account for a very small fraction of the channels expressed at the cell surface.

The binding assay also allowed us to estimate the relative abundance of each $\alpha$, $\beta$, and $\gamma$ subunit expressed at the cell surface. Table I shows the specific binding of $^{[125]}$I-anti-FLAG mAb normalized per $\mu$A of $I_{Na}$ (fmol/$\mu$A) in individual oocytes expressing different combinations of FLAG-tagged subunits. Among oocytes expressing channels made of one FLAG-tagged subunit and two untagged subunits, a higher binding signal was detected when the FLAG epitope was present on the $\alpha$ subunit ($\alpha^F\beta\gamma$) compared with conditions where the epitope was on the $\beta$ or $\gamma$ subunits ($\alpha\beta^F\gamma$, $\alpha\beta\gamma^F$). These binding values obtained for channels with one tagged subunit suggest that the relative abundance of ENaC subunits at the cell surface follows the order $\alpha > \beta > \gamma$, although the difference in binding between oocytes expressing $\alpha\beta^F\gamma$ and $\alpha\beta\gamma^F$ did not reach statistical significance ($P = 0.06$). Comparison of oocytes expressing channels made of two FLAG-tagged subunits and one untagged subunit shows similar results. A higher binding signal (fmol/$\mu$A) was measured when the $\alpha$ subunit was tagged ($\alpha^F\beta\gamma^F$, $\alpha^F\beta\gamma^F$) compared with channels with tagged $\beta$ and $\gamma$ subunits ($\alpha\beta^F\gamma^F$). The higher number of $\alpha$ subunits per active channels detected at the cell surface suggests that the subunit composition of ENaC involves significantly more $\alpha$ than $\beta$ or $\gamma$ subunits, and consequently that the channel stoichiometry consists of more than three subunits. However, the information obtained from these latter binding experiments is limited in terms of absolute numbers of subunits forming the channel. We have therefore used two

<table>
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<th>Binding (fmol/$\mu$A)</th>
<th>$P$</th>
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<tr>
<td>$\alpha^F\beta\gamma$</td>
<td>0.055 ± 0.007</td>
<td>0.006</td>
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<tr>
<td>$\alpha\beta^F\gamma$</td>
<td>0.028 ± 0.005</td>
<td>0.062</td>
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<tr>
<td>$\alpha\beta\gamma^F$</td>
<td>0.016 ± 0.002</td>
<td>0.006</td>
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<tr>
<td>$\alpha^F\beta^F\gamma$</td>
<td>0.044 ± 0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>$\alpha\beta^F\gamma^F$</td>
<td>0.085 ± 0.009</td>
<td>0.004</td>
</tr>
<tr>
<td>$\alpha\beta\gamma^F$</td>
<td>0.105 ± 0.007</td>
<td>0.104</td>
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<tr>
<td>$\alpha^F\beta^F\gamma^F$</td>
<td>0.156 ± 0.018</td>
<td>0.104</td>
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Specific binding of $^{[125]}$I-anti-FLAG antibody (12 nM) normalized for $I_{Na}$ (fmol/$\mu$A) in oocytes expressing combinations of one FLAG-tagged subunit together with two non-tagged subunits ($\alpha^F\beta\gamma$, $\alpha\beta^F\gamma$, $\alpha\beta\gamma^F$) and combinations of two FLAG-tagged subunits with one non-tagged subunit ($\alpha\beta^F\gamma^F$, $\alpha\beta\gamma^F$, $\alpha\beta\gamma^F$). Oocytes were injected with equal amounts (3.3 ng) of $\alpha$, $\beta$, and $\gamma$ subunit cRNAs. The equilibrium dissociation constant $K_d$ of the antibody was similar for the three FLAG-tagged subunits (3 nM) as determined in separate experiments (data not shown). Results are means ± SE for $n$ independent experiments each including 8–10 oocytes. Specific binding of $^{[125]}$I-anti-FLAG M2 monoclonal antibodies to FLAG-tagged ENaC subunits was performed in individual oocytes according to Firsov et al. (1996).
other independent approaches to assess ENaC subunit stoichiometry.

**Subunit stoichiometry assessed by interactions of ligands with co-expressed sensitive and resistant mutant subunits**

Channel subunit stoichiometry can, alternatively, be determined by functional analysis of differential channel sensitivities to blockers conferred by point mutations as first described by MacKinnon (1991) for the Shaker K channel. The Cys substitutions for residues Gly525 and Gly537 in the $\beta$ and $\gamma$ ENaC subunits respectively ($\beta_{G525C}$ and $\gamma_{G537C}$ mutants) decrease ENaC affinity for amiloride by >1000-fold (Schild et al., 1997). The corresponding Ser583→Cys mutation on the $\alpha$ subunit ($\alpha_{S583C}$) exhibited a unique channel block by external Zn$^{2+}$ (Schild et al., 1997). The stoichiometry of the $\alpha$ subunit was first assessed by blocker titration curves of channel mixtures resulting from co-expression of fixed ratios of $\alpha$ wild-type and $\alpha_{S583C}$ mutant subunits with wild-type $\beta$ and $\gamma$ subunits. Figure 2A shows that wild-type ENaC is insensitive to block by Zn$^{2+}$ (inhibitory constant $K_i > 10 \text{ mM}$), whereas the $\alpha_{S583C}$ mutant is highly sensitive to Zn$^{2+}$ block ($K_i = 30 \text{ mM}$). The inhibition curves obtained for mixtures of wild-type and mutant $\alpha$ subunits are distinctly biphasic and could well be described by the participation of two channel types, i.e. Zn$^{2+}$-sensitive and Zn$^{2+}$-insensitive channels, with $K_i$s for Zn$^{2+}$ block of 30 $\text{ mM}$ and 16 $\text{ mM}$ respectively. Indeed, a Zn$^{2+}$ titration curve will not show a biphasic behaviour, as in Figure 2A, if a significant fraction of channels with intermediate Zn$^{2+}$ sensitivity is generated by the expression of mixtures of wild-type and mutant $\alpha$ subunits.

For a fractional expression of the $\alpha_{S583C}$ mutant ($f_{mut}$) of 0.43, only a small fraction of channels expressed are sensitive to Zn$^{2+}$ block, as shown in Figure 2A by the 20% inhibition of $I_{Na}$ obtained with 1 $\text{ mM}$ Zn$^{2+}$. On increasing the fractional expression of the $\alpha_{S583C}$ mutant to 0.79 ($f_{mut}$), only 60% of amiloride-sensitive current shows a high sensitivity to block by Zn$^{2+}$. These results indicate (i) that expression of mixtures of $\alpha$ wild-type and $\alpha_{S583C}$ mutant subunits produced two distinct populations of channels with Zn$^{2+}$ blocking affinities of either fully wild-type or $\alpha_{S583C}$ mutant channel phenotype, and (ii) that Ser583 of the wild-type $\alpha$ subunit has a dominant effect on resistance to Zn$^{2+}$ block, suggesting that more than one mutated subunit is needed to confer Zn$^{2+}$ sensitivity to the channel. Using MacKinnon’s formalism, the titration curves for wild-type and $\alpha_{S583C}$ mutant channels were best fitted assuming the participation of two $\alpha$ subunits in conferring Zn$^{2+}$ sensitivity (Figure 2B). The predictions obtained for alternative subunit stoichiometries, such as three $\alpha$ subunits where all three or only two mutated subunits confer Zn$^{2+}$ sensitivity, significantly deviate from our experimental data.

The amiloride titration curves of $I_{Na}$ in oocytes expressing mixtures of wild-type $\beta$ and $\beta_{G525C}$ mutant subunits together with $\alpha$ and $\gamma$ wild-type subunits are also biphasic. The amiloride-sensitive component of $I_{Na}$ exhibits a $K_i$ similar to wild-type, while the amiloride-resistant component with a $K_i$ in the micromolar range is comparable with the fully $\beta_{G525C}$ mutant channel (Figure 3A). The amiloride-resistant $I_{Na}$ plateaus at a fraction of $0.3$ and $0.7$ of the total current, values that correspond to the fractional expression of the $\beta_{G525C}$ channel mutant ($f_{mut} = 0.32$ and $0.7$). Thus no dominant phenotype regarding amiloride sensitivity can be observed for the $\beta_{G525C}$-mutation, by contrast to the dominant Zn$^{2+}$-resistant phenotype shown previously. These findings are expected for two distinct amiloride-sensitive and -resistant channels, with a single $\beta_{G525C}$ mutation on one $\beta$ subunit responsible
for the amiloride resistance. A single β subunit stoichiometry predicts an amiloride titration curve that closely fits our experimental results (Figure 3A). Alternative models involving the participation of two βG525C mutations on two β subunits responsible for channel resistance to amiloride could not fit our experimental results (data not shown).

Amiloride inhibition curves obtained from expression of mixtures of wild-type γ and γG537C mutant subunits exhibit a similar biphasic shape to that observed for the βG525C mutant, indicative of the presence of two types of channels, either sensitive or resistant to amiloride (Figure 3B). The fraction of amiloride-resistant I_Na is related directly to the fraction of γG537C mutant channels expressed (fmut = 0.64 and 0.26), indicating no dominant or recessive effect of the mutation. The amiloride inhibition curves (solid line) for mixtures of wild-type γ and γG537C mutant channels are consistent with a model of one but not two γ subunits involved in amiloride resistance (Figure 3B).

Subunit stoichiometry assessed with concatameric αβγ constructs

Analysis of Zn^{2+} and amiloride block of I_Na resulting from expression of mixtures of wild-type and mutant subunits suggests a minimal ENaC stoichiometry of two α, one β and one γ subunit. It remains possible that the number of channel subunits determined from Zn^{2+} and amiloride blocking interactions with the channel might be different from the actual ENaC subunit stoichiometry. In order to obtain further evidence for a two α, one β, one γ stoichiometry, we have used an additional approach based on the generation of different concatamers (tri- and tetrameric constructs) made of α, β and γ subunit cDNAs linked in a head-to-tail fashion (Liman et al., 1992). These trimeric and tetrameric constructs were co-expressed with monomeric α, β or γ subunits to test for complementation of the concatamers by mutant or wild-type monomeric subunits in forming functional channels. Complementation was assessed by changes in the pharmacological properties of the channel. This strategy assumes that single monomeric subunits cannot form a homomeric channel with detectable I_Na; this assumption was verified in previous experiments showing that at most 1% of I_Na could be accounted for by functional homomeric channels resulting from expression of the α subunit alone, whereas no current is detected with expression of β or γ subunits (Canessa et al., 1994b; Firsov et al., 1996).

To ascertain first whether efficient translation and correct protein synthesis occurred, cRNAs encoding concatamers were co-injected with monomeric α subunit cRNA into *Xenopus* oocytes which had been metabolically labelled with [35S]methionine. Expressed channels were then immunoprecipitated with an anti-α polyclonal antibody. The experiments in Figure 4 with expression of β–γ–α and β–γ–α–α concatamers show that the polypeptides are correctly synthesized, as evidenced by proteins with the expected 250 and 340 kDa mol. wts respectively. The translation of the tri- and the tetrameric concatamers was ~10 time less efficient than that of the monomeric α subunit (95 kDa). Similar results were obtained with the other tri- or tetrameric constructs tested in this study. The lower efficiency of concatamer translation required, for the complementation experiments, injection of a 10-fold lower quantity of message encoding monomeric subunits.

Expression of the mutant trimeric construct β–γ–αG583C made from the αG583C mutant subunit and wild-type β and γ subunits, generated a robust amiloride-sensitive Na^+ current. The current expressed was sensitive to Zn^{2+} at a concentration of 1 mM. This significant fraction of Zn^{2+}-resistant current indicates that the monomeric α subunit is capable of complementing the trimer, and in doing so renders half of the channels resistant to Zn^{2+}...
blockade. The interpretation of these results is illustrated in Figure 6A. When expressed alone, the β–γ–αS583C trimer is complemented by a αS583C subunit that is part of a separate polypeptide chain, and the two mutated α subunits confer Zn²⁺ sensitivity to the channel. When co-expressed with monomeric α subunits, complementation of the β–γ–αS583C mutant trimer by a wild-type α subunit results in hybrid channels composed of one wild-type α and one αS583C mutant subunit that are insensitive to Zn²⁺ in accordance with the dominant Zn²⁺-resistant phenotype shown previously. The wild-type β–γ–α trimer is insensitive to Zn²⁺, and co-expression with the αS583C mutant subunit does not change channel resistance to Zn²⁺, as expected for hybrid channels made of one wild-type and one mutant α subunit. The same results were obtained with different wild-type α–γ–β, α–β–γ and β–α–γ trimer constructs co-expressed with the αS583C mutant subunit (data not shown). The evidence that the β–γ–αS583C trimer is complemented by α subunits is consistent with a channel made of at least two α subunits, and further supports the hypothesis that channel blockade by Zn²⁺ involves coordination with two cysteines provided by two distinct α subunits. Furthermore, we can exclude the possibility that three α subunits are involved in the formation of the channel pore: if wild-type trimers are complemented by two or more monomeric αS583C mutants, this should have conferred, at least to some channels, the Zn²⁺-sensitive phenotype that requires two cysteines on two α subunits. No Zn²⁺-sensitive current could be detected in co-expression experiments of wild-type β–γ–α, α–γ–β, α–β–γ or β–α–γ trimers with αS583C mutant subunits, indicating that these trimers are indeed complemented by a single αS583C mutant subunit.

Expression of β–γ–α trimers resulted in amiloride-sensitive channels with an amiloride affinity identical to that of channels expressed by monomeric α, β and γ subunits (Figures 3A and B, and 5B and C). Co-expression of the β–γ–α trimer with either βG525C or γG537C mutants did not significantly modify channel sensitivity to amiloride, indicating that monomeric β and γ mutants do not comple-

Fig. 4. Immunoprecipitation profile of co-expressed β–γ–α or β–γ–α–α cDNA constructs together with the α ENaC subunit. Oocytes were injected with β–γ–α or β–γ–α–α cRNAs with α cRNA in a molar ratio of either 1:1 or 10:1 respectively.

Fig. 5. Complementation of tandem trimeric β–γ–α constructs by monomeric α, β or γ subunits. (A) Zn²⁺ titration curve of Na⁺ current (Iₙa) expressed by the wild-type trimeric construct β–γ–α alone (●, mean Iₙa = 32 ± 12 μA) or co-expressed with the αS583C subunit (■, mean Iₙa = 15 ± 6 μA), and the β–γ–αS583C trimer (Δ, mean Iₙa = 28 ± 12 μA) alone or with α wild-type subunit (∆, mean Iₙa = 15 ± 9 μA). Fit of the data gave a Kᵢ of 100 μM for Zn²⁺ block of the β–γ–αS583C. In oocytes expressing β–γ–αS583C together with the α wild-type subunit, 50% of Iₙa is insensitive to Zn²⁺ block at a Zn²⁺ concentration of 1 mM. (B) Amiloride inhibition curve of Iₙa expressed by the wild-type trimeric construct β–γ–α (●, mean Iₙa = 6 ± 3.5 μA) alone or together with the βG525C subunit (■, mean Iₙa = 2.5 ± 1.6 μA) shows a Kᵢ of 86 and 33 nM respectively. The amiloride Ki for the trimer βG525C–γ–α (□, mean Iₙa = 5.2 ± 4.1 μA) was 58 and 31 μM when co-expressed with the wild-type β (●, mean Iₙa = 3.9 ± 2.9 μA). (C) Amiloride inhibition curve of Iₙa expressed by the wild-type β–γ–α trimer (●, mean Iₙa = 11 ± 9 μA, Kᵢ = 49 nM) or co-injected with mutant γG537C subunits (■, mean Iₙa = 6.2 ± 5 μA, Kᵢ = 75 nM). The trimeric mutant β–γG537C–α (Δ, mean Iₙa = 13 ± 6 μA) was resistant to amiloride (Kᵢ = 62 μM) when expressed alone or together with the γ wild-type subunit (△, mean Iₙa = 8.6 ± 4 μA, Kᵢ = 21 μM). Each data point represents the mean ± SE of 8–12 oocytes. Data were fitted to a Langmuir isotherm (solid lines). * Denotes mutated subunits, αS583C, βG525C or γG537C.
complementation experiments of ENaC subunit trimers further support a stoichiometry consisting of two α, one β and one γ subunit. They do not, however, provide any information on the subunit arrangement around the channel pore.

Complementation of the trimeric βγα construct with monomeric α subunits may result in two distinct subunit arrangements with respect to the position of α subunits, as illustrated in Figure 6. In one configuration, the two α subunits are located side by side flanked by β and γ subunits (Figure 6A). The other configuration places the two α subunits opposite each other across the channel pore, separated by β and γ subunits (Figure 6B). To explore these two alternative subunit arrangements, βγα−γ and βγα−α−α tetramers were constructed with the αS583C mutation on both subunits. When expressed, both tetramers generated a robust amiloride-sensitive Na⁺ current that was blocked by Zn²⁺ with a Kᵢ of 42 μM (Figure 7A). Co-expression of the αS583CβαS583C−γ tetrameric construct with monomeric wild-type α subunits did not change the Zn²⁺ sensitivity expressed by the tetramer alone. It indicates that the αS583CβαS583C−γ tetramer does not require complementation by another α subunit to form a functional channel, consistent with a two α subunit stoichiometry. By contrast, the βγα−αS583C−αS583C tetramer was complemented by a monomeric α subunit, as determined by a complete loss of channel sensitivity to Zn²⁺ block when the tetramer was co-expressed with wild-type subunits. Complementation of the γαS583C−αS583C−β tetramer by α monomers could also be evidenced (data not shown). Thus only one α subunit of the βγα−αα or γ−α−α−β tetramers participates in the channel pore, the other α subunit being supplied by either a separate tetramer or a monomeric α subunit, in forming the correct subunit arrangement around the channel pore. The absence of complementation of the αS583C−β−αS583C−γ tetramer by α subunit favours the picture of ENaC being comprised of two α subunits separated by β and γ subunits arranged in a ring-like structure around the channel pore. The ENaC stoichiometry and subunit arrangement of ENaC is supported further by the experiments shown in Figure 7B. This tetramer formed with one mutant αS583C and one wild-type α subunit is resistant to Zn²⁺ block, confirming our previous observation, made with co-expression of mixtures of α subunits, that Zn²⁺ blockade requires two mutated αS583C subunits. Co-expression of the α−β−αS583C−γ tetramer with a mutated αS583C subunit did not generate Zn²⁺-sensitive channels, indicating that the α−β−αS583C−γ tetramer is not complemented by the monomeric αS583C subunit. Finally, we have verified at the single
channel level that the $\alpha$-$\beta$-$\alpha$-$\gamma$ tetramer reproduces the biophysical and pharmacological properties of the wild-type ENaC: a slow gating channel with openings and closures on the time scale of 1 s; a 5 pS conductance for Na$^+$ ions; and a typical ionic selectivity sequence of Li$^+$ > Na$^+$ >> K$^+$ (data not shown).

**Discussion**

In the absence of high resolution images of the ENaC, its subunit stoichiometry can be assessed alternatively by indirect approaches consisting of tagging individual subunits with radiolabelled antibodies or using site-directed mutations that modify the functional properties of the channel. In the present work, we have combined three different approaches to determine the number of $\alpha$, $\beta$ and $\gamma$ subunits that participate in the formation of the ion-conducting structure of the channel. A quantitative analysis of cell surface expression of ENaC $\alpha$, $\beta$ and $\gamma$ subunits enabled us to show that ENaC subunits assemble according to a fixed stoichiometry, and that the number of $\alpha$ subunits normalized per functional channel expressed at the cell surface is higher than $\beta$ or $\gamma$ subunits. Two functional assays, based on differential sensitivities to channel blockers elicited by mutations on individual subunits, and on the functional expression of concatameric ENaC polypeptides, provide evidence that ENaC is a tetramer made of two $\alpha$, one $\beta$ and one $\gamma$ subunit that assemble pseudosymmetrically around the channel pore to form a functional channel. In addition, complementation of tetrameric concatamers allowed us to provide evidence that the likely subunit configuration around the channel pore consists of two $\alpha$ subunits separated by one $\beta$ and one $\gamma$ subunit.

In the *Xenopus* oocyte expression system, the $\alpha$, $\beta$ and $\gamma$ subunits are necessary for maximal expression of functional channel complexes. Previous reports have shown that individual $\beta$ or $\gamma$ subunits by themselves are not expressed at the plasma membrane and cannot form a functional channel (Canessa et al., 1994b; Firsov et al., 1996). In the initial cloning experiments, expression of ENaC $\alpha$ subunits resulted in a small current amplitude that represents at most 1% of the amiloride-sensitive current resulting from co-expression of $\alpha$, $\beta$ and $\gamma$ subunits (Canessa et al., 1993). This barely detectable amiloride-sensitive current resulting from expression of $\alpha$ subunits alone correlates with the low level of $\alpha$ subunits present at the cell surface as if $\alpha$ subunits were not efficiently targeted to the plasma membrane in the absence of $\beta$ and $\gamma$ subunits (Firsov et al., 1996). Therefore, the presence of individual ENaC subunits or homomeric channels at the cell surface will not interfere to a significant extent with our results. Co-expression of two ENaC subunits, either $\alpha$ together with $\beta$ or $\alpha$ with $\gamma$ subunits, generates functional amiloride-sensitive channels with slightly different functional properties (McNicholas and Canessa, 1997). Once again, the level of expression of these $\alpha\beta$ or $\alpha\gamma$ channel complexes at the cell surface is low, and the amiloride-sensitive current measured represents at most 5% of that measured with co-expression of the three $\alpha$, $\beta$ and $\gamma$ subunits. When the $\alpha$, $\beta$ and $\gamma$ subunits are co-expressed, we have been able to show that the subunits in the $\alpha\beta\gamma$ channel complex are not interchangeable, implying a preferential assembly that involves $\alpha$, $\beta$ and $\gamma$ subunits. The preferred $\alpha\beta\gamma$ form of ENaC is not altered by injection of a 100-fold higher quantity of message encoding a single subunit. Thus, in *Xenopus* oocytes, differential assembly of ENaC subunits into various configurations upon co-expression of $\alpha$, $\beta$ and $\gamma$ subunits is unlikely to occur. This is supported further by our complementation experiments with tetrameric constructs which provide evidences that the subunit arrangement around the channel pore forms a preferred $\alpha\beta\alpha\gamma$ configuration. A similar preferential subunit assembly has been documented for the $\alpha_1\beta_1\gamma_2$ subunits of the GABA$_A$ receptor channel: upon expression of the $\alpha_1$, $\beta_1$ and $\gamma_2$ subunits, assembly results in a preferred $\alpha_1\beta_1\gamma_2$ form of the channel and the functional $\alpha_1\beta_1$ complex is almost never observed at the cell surface (Angelotti and Macdonald, 1993). The preferential ENaC subunit assembly shown in the frog oocytes does not, however, exclude the possibility that in native tissues where two subunits are expressed predominantly, as, for instance, in the lung, ENaC channels might exist in different subunit compositions (Farman et al., 1997). If ENaC exists in different subunit configurations, the tetrameric architecture of the channel will certainly be conserved, as well as the number of $\alpha$ subunits, leading to $\alpha_2\beta_1$ or $\alpha_2\gamma_2$ channel complexes.

The subunit stoichiometry of the other members of the ENaC/DEG channel gene superfamily, such as BNaC or degenerins, has not yet been established. However, genetic experiments on *C. elegans* suggest channel assembly from two different but homologous degenerin genes *MEC-4* and *MEC-10*, and the presence of more than one *MEC-4* subunit in forming the mechanotransducing channel complex (Huang and Chalfie, 1994; Du et al., 1996). Because of similarities in their primary and secondary structure and in their functional and pharmacological characteristics, we propose that members of the ENaC/DEG gene superfamily share a common heterotetrameric structure. The tetrameric subunit stoichiometry represents a structural feature common to other cation-selective channels belonging to the voltage/second messenger-gated channel family and to other eukaryotic potassium channel families such as the inward rectifier and slowpoke K channels. These channel proteins contain four homologous domains that associate to form the channel pore. The pore region (P region) involves a hairpin structure flanked by two transmembrane $\alpha$ helices that enters and leaves the membrane at the extracellular side.

The structures of ENaC involved in the ion permeation pathway have not yet been defined clearly. However, the Ser583 residue on the $\alpha$ subunit and the corresponding Gly525 and Gly537 residues on the $\beta$ and $\gamma$ subunits are clearly located within the ion permeation pathway since cysteine substitutions at these positions generates a Zn$^{2+}$-binding site that either blocks the channel or reduces the single channel conductance (Schild et al., 1997). The domain encompassing $\alpha$Ser583, $\beta$Gly525 and $\gamma$Gly537 may well represent a similar hairpin structure preceding a transmembrane $\alpha$ helix involved in the outer mouth of the channel pore. Consistent with this model, experiments using proteolytic digestion of $\alpha$ENaC have demonstrated that a short segment encompassing Ser583 in the $\alpha$ subunit was insensitive to proteolysis, suggesting an intramembrane topology (Renard et al., 1994).
Thus, the picture of K or Na channels that arose from numerous structure–function studies, i.e. a membrane protein formed by four subunits arranged in a ring-like structure around a central pore, also seems to apply to ENaC and probably to all the members of the ENaC/degenerin gene family. Despite their unrelated primary sequences, ENaC and other cation channels such as inward rectifier K channels and Shaker K channels may represent a diverse array of related proteins, in particular with respect to the structure forming the channel pore.

Materials and methods

Binding experiments

Specific binding of $[^{125}\text{I}]$-anti-FLAG M2 mAbs to FLAG-tagged ENaC subunits was performed in individual oocytes as previously reported (Firsov et al., 1996).

Heterologous expression of wild-type and mutant ENaC subunits

The experiments were performed with the rat ENaC cDNA encoding $\alpha$, $\beta$ and $\gamma$ subunits. Mutations were introduced in the cDNA sequence by PCR using a three-step protocol described previously (Schild et al., 1997). Complementary RNAs of each $\alpha$, $\beta$ and $\gamma$ subunit were synthesized in vitro and stored at a concentration of 100 ng/$\mu$L. Oocytes were injected with 10 ng of cRNA encoding each $\alpha$, $\beta$ and $\gamma$ subunit (total cRNA = 10 ng), unless otherwise stated in the figure legends. This cRNA concentration is ~10-fold higher than the lowest concentration (1 ng of cRNA) able to produce maximal expression of ENaC channels.

In oocytes expressing a mixture of blocker-sensitive and -insensitive subunits, the overall unblocked fraction ($U_{\text{max}}$) was recorded at a holding potential of ~100 mV as previously described (Schild et al., 1995).

For oocytes expressing a mixture of blocker-sensitive and -insensitive subunits, the overall unblocked fraction ($U_{\text{max}}$) at a holding potential of ~100 mV was recorded as previously described (Schild et al., 1995).

$$U_{\text{max}} = \sum_{i=0}^{n} F_i \left( \frac{K_i}{K_i + [B]} \right)$$

where $K_i$ is the inhibition constant for the $i$th channel species and $P_i$ is the fraction of channels that are $i$-type (sensitive or resistant). $F_i$ is given by:

$$F_i = \left( \sum_{j=0}^{n} f_{\text{mut}}^j \right) - f_{\text{mut}}^i$$

where $f_{\text{mut}}$ and $f_{\text{wt}}$ are respectively the fraction of mutant and wild-type $\alpha$ subunits expressed by the oocyte, with n the number of $\alpha$ subunits.

The fractions $f_{\text{mut}}$ and $f_{\text{wt}}$ were determined by known ratios of CRNAs as assumed that wild-type and mutant subunits are expressed to the same extent and assemble in a random fashion. $f_{\text{mut}}$ expressed by the $\alpha_{555\times\beta\gamma}$ construct, $\alpha_{G525\times\gamma\gamma}$ and $\alpha_{G537\times\gamma\gamma}$ mutants were respectively 0.76 ± 0.13, 0.47 ± 0.07 and 1.50 ± 0.18 ($n = 45–55$ oocytes) relative to wild-type ENaC. Thus, for a 1:1 cRNA mixture of $\alpha_{555\times\beta\gamma}$ mutant and wild-type $\alpha$, the fractional expression of the mutant channel types is lower than expected from the amount of cRNA injected, and $f_{\text{mut}}$ becomes 0.43 (0.76:1) instead of 0.5 (1:1). Similarly, a 1:1 cRNA mixture of $\alpha_{G525\times\gamma\gamma}$ mutant and wild-type $\alpha$ effectively corresponds to a $f_{\text{mut}}$ value of 0.32 (0.47:1) instead of 0.5 (1:1). A 1:1 cRNA mixture of $\alpha_{G537\times\gamma\gamma}$ mutant over wild-type $\alpha$ is not expressed effectively in the 1:1 mixture of $\alpha_{555\times\beta\gamma}$ wild-type, $\beta_{G525\times\gamma\gamma}$ wild-type and $\gamma_{G537\times\gamma\gamma}$ wild-type cRNAs (data not shown).

Construction and expression of concatameric cDNA constructs

Concatameric constructs have been produced by linking the coding sequences of ENaC subunits in a head-to-tail fashion. Two types of ENaC subunit construct were used: one called ’3-modified construct’ in which the stop codons were mutated to contain a unique HpaI restriction endonuclease coding sequence (GGT/AAC) followed by a unique XhoI site in the 3’ non-coding region. The other so-called ’5’-3’-modified construct’ contained the 3’ construct modifications described above in addition to replacement of the first 5’ ATG codon with a linker of eight glutamines as an inter-subunit bridge; in addition, a unique EcoRV restriction site has been introduced preceding the first 5’ glutamine codon site. All the constructs have been verified by DNA sequencing.

Multimeric constructs were obtained by digestion of the ’5’-3’-modified construct’ with EcoRV and XhoI and insertion of the digestion product into the ’3’-modified construct’ cut with HpaI–XhoI.

To check for correct protein synthesis, cRNAs encoding the trimeric or tetrameric constructs were injected into oocytes metabolically labelled with $[^{35}$S]methionine and immunoprecipitated with anti-α rabbit polyclonal antibody. In co-expression experiments, oocytes were injected with 10 ng of cRNA encoding the trimeric or tetramer and 1 ng of cRNA encoding the monomeric subunit to obtain equal amounts of synthesized protein.

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