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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 the four subunit channel stoichiometry and showed 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 pseudohypoaldosteronism 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 appproach 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 quantitate cell surface expression of ENaC subunits in intact oocytes (Firsov *et al.*, 1996). Pairs of subunits were tagged with a FLAG epitope (either $\beta^F\gamma^F$, $\alpha^F\gamma^F$ or $\alpha^F\beta^F$) and co-expressed with increasing cRNA concentrations of the untagged subunits (Figure 1A–F). In individual oocytes, specific binding of [^{125}I]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 (I_{Na}) (Figure 1A, C and E) (Firsov *et al.*, 1996). This linear correlation allows us to normalize the binding signal (in fmol) per μA of I_{Na}, 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

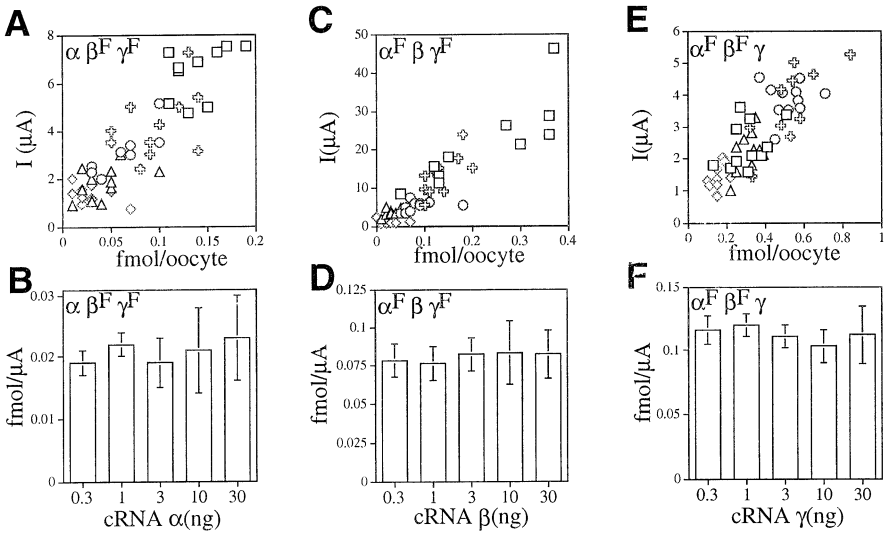

Fig. 1. ENaC expressed at the cell surface is composed of a fixed number of α, β and γ subunits. (A and B) *Xenopus* oocytes were injected with 0.3 ng of cRNA of each FLAG-tagged β and γ ENaC subunit (β^F,γ^F) and different cRNA concentrations [0.3 (□), 1 (⊡), 3 (○), 10 (△) and 30 (◇) ng] of the non-tagged α subunit. (**A**) A representative experiment showing the linear correlation between binding of [^{125}I]anti-FLAG antibodies to β and γ FLAG-tagged subunits (fmol/oocyte) and the amiloride-sensitive current I_{Na} measured in individual oocytes. (**B**) Ratios of specific antibody binding normalized for I_{Na} (fmol/μA) measured in individual oocytes over a wide range of injected α non-tagged cRNA concentrations. Errors bars are means ± SE of three experiments performed with 12 oocytes per experimental condition. (**C** and **D**) Same experiments as in (A) and (B) but with oocytes injected with 0.3 ng of cRNA of FLAG-tagged α and γ ENaC subunits (α^F,γ^F) and increasing cRNA concentrations [0.3 (□), 1 (⊡), 3 (○), 10 (△) and 30 (◇) ng] of the non-tagged β subunit. (**E** and **F**) Same experiments as in (A) and (B) but with oocytes injected with 0.3 ng of cRNA of FLAG-tagged α and β ENaC subunits (α^F,β^F) and increasing cRNA concentrations [0.3 (□), 1 (⊡), 3 (○), 10 (△) and 30 (◇) ng] of the non-tagged γ subunit.

determined by specific binding of [^{125}I]anti-FLAG mAb normalized for the measured current I_{Na} (fmol/μA) remains constant, despite injection of increasing concentrations of cRNA encoding the untagged subunit. Thus when the three subunits are co-expressed, α, β and γ 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 α, β and γ subunits with similar concentrations of cRNA message, the formation of αβ or αγ 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 α, β and γ subunit expressed at the cell surface. Table I shows the specific binding of [^{125}I]anti-FLAG mAb normalized per μA of I_{Na} (fmol/μA) 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 α subunit ($\alpha^F\beta\gamma$) compared with conditions where the epitope was on the β or γ 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/μA) was measured when the α subunit was tagged ($\alpha^F\beta^F\gamma$, $\alpha^F\beta\gamma^F$) compared with channels with tagged β and γ subunits ($\alpha\beta^F\gamma^F$). The higher number of α subunits per active channels detected at the cell surface suggests that the subunit composition of ENaC involves significantly more α than β or γ 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 I. Differential expression of α, β and γ ENaC subunits at the cell surface

	Binding (fmol/μA)	*P*	*n*
$\alpha^F\beta\gamma$	0.055 ± 0.007		10
		0.006	
$\alpha\beta^F\gamma$	0.028 ± 0.005		10
		0.062	
$\alpha\beta\gamma^F$	0.016 ± 0.002		8
$\alpha\beta^F\gamma^F$	0.044 + 0.006		10
		0.002	
$\alpha^F\beta\gamma^F$	0.085 ± 0.009		10
		0.104	
$\alpha^F\beta^F\gamma$	0.105 ± 0.007		10
$\alpha^F\beta^F\gamma^F$	0.156 ± 0.018		10

Specific binding of [^{125}I]anti-FLAG antibody (12 nM) normalized for I_{Na} (fmol/μA) in oocytes expressing combinations of one FLAG-tagged subunit together with two non-tagged subunits (α^F–β–γ, α–β^F–γ, α–β–γ^F) and combinations of two FLAG-tagged subunits with one non-tagged subunit (α–β^F–γ^F, α^F–β–γ^F, α^F–β^F–γ). Oocytes were injected with equal amounts (3.3 ng) of α, β and γ 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 β and γ ENaC subunits respectively (β_{G525C} and γ_{G537C} mutants) decrease ENaC affinity for amiloride by >1000-fold (Schild *et al.*, 1997). The corresponding Ser583→Cys mutation on the α subunit (α_{S583C}) exhibited a unique channel block by external Zn^{2+} (Schild *et al.*, 1997). The stoichiometry of the α subunit was first assessed by blocker titration curves of channel mixtures resulting from co-expression of fixed ratios of α wild-type and α_{S583C} mutant subunits with wild-type β and γ subunits. Figure 2A shows that wild-type ENaC is insensitive to block by Zn^{2+} (inhibitory constant K_i >10 mM), whereas the α_{S583C} mutant is highly sensitive to Zn^{2+} block (K_i = 30 μM). The inhibition curves obtained for mixtures of wild-type and mutant α 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_is for Zn^{2+} block of 30 μM and 16 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 α subunits.

For a fractional expression of the α_{S583C} mutant (*fmut*) 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 mM Zn^{2+}. On increasing the fractional expression of the α_{S583C} mutant to 0.79 (*fmut*), only 60% of amiloride-sensitive current shows a high sensitivity to block by Zn^{2+}. These results indicate (i) that expression of mixtures of α wild-type and α_{S583C} mutant subunits produced two distinct populations of channels with Zn^{2+} blocking affinities of either fully wild-type or α_{S583C} mutant channel phenotype, and (ii) that Ser583 of the wild-type α 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 α_{S583C} mutant channels were best fitted assuming the participation of two α subunits in conferring Zn^{2+} sensitivity (Figure 2B). The predictions obtained for alternative subunit stoichiometries, such as three α 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 β and β_{G525C} mutant subunits together with α and γ 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 β_{G525C} mutant channel (Figure 3A). The amiloride-resistant I_{Na} plateaus at a fraction of

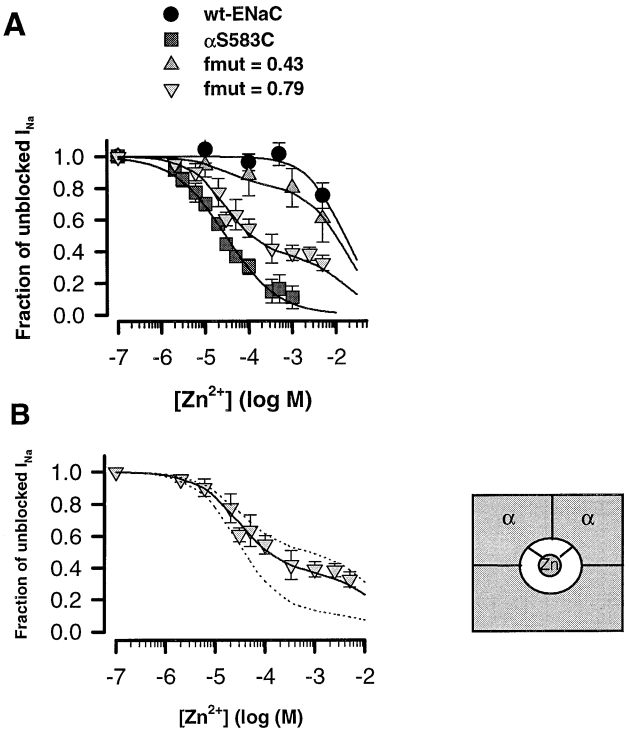

Fig. 2. Zn^{2+} inhibition curves of Na^+ current expressed by the αβγ ENaC using wild-type and/or mutant α subunits. (**A**) Wild-type αβγ ENaC (●) is resistant whereas the $\alpha_{S583C}\beta\gamma$ mutant (■) is sensitive to block by Zn^{2+}. Fit of the data to the Langmuir isotherm (solid line) gives a K_i of 16 mM for the wild-type ENaC and a K_i of 30 μM for the mutant $\alpha_{S583C}\beta\gamma$. Expression of mixtures of α_{S583C} and α wild-type (▲, ▽) together with β and γ subunits shows a biphasic Zn^{2+} inhibition curve. Data from fractional expression (f_{mut}) of the $\alpha_{S583C}\beta\gamma$ mutant of 0.43 (▲) and 0.79 (▽) over wild-type α subunits co-expressed with β and γ subunits were fitted to the sum of two Hill equations (solid lines):

$$\frac{I}{I_{con}} = I_1 \left\{ 1 + \left(\frac{B}{K_{i1}} \right) \right\} + I_2 \left\{ 1 + \left(\frac{B}{K_{i2}} \right) \right\}$$

where B is the Zn^{2+} concentration, I_1 and I_2 are the fractional current of the Zn^{2+}-sensitive and Zn^{2+}-resistant components of I_{Na}, and K_{i1} and K_{i2} their inhibition constants of 30 and 16 mM. Error bars are means ± SE. (**B**) Predictions for the number of mutated α subunits required to confer Zn^{2+} sensitivity on the channel. Data are taken from (A). The solid line represents the prediction for a two α subunit stoichiometry where each subunit participates in Zn^{2+} coordination as depicted by the schematic representation of the ENaC channel. Fit was obtained according to Equations 1 and 2 in Materials and methods. Fit of the data (solid line) assumes n = 2 α subunits, a Zn^{2+} K_i of 30 μM for the mutant channel with both α subunits mutated (α_{S583C}–α_{S583C}) and a K_i of 16 mM for the Zn^{2+}-resistant channels including the fully wild-type ENaC ($\alpha_{wild\text{-}type}$–$\alpha_{wild\text{-}type}$) and the hybrid channels ($\alpha_{wild\text{-}type}$–$\alpha_{S583C}$ and α_{S583C}–$\alpha_{wild\text{-}type}$) having one α subunit mutated. The dotted lines represent the predictions for a channel made of three α subunits in which either all three mutated subunits (upper dotted line) or only two mutated subunits (lower dotted line) are necessary to confer Zn^{2+} sensitivity.

~0.3 and 0.7 of the total current, values that correspond to the fractional expression of the β_{G525C} channel mutant (*fmut* = 0.32 and 0.7). Thus no dominant phenotype regarding amiloride sensitivity can be observed for the β_{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 β_{G525C} mutation on one β subunit responsible

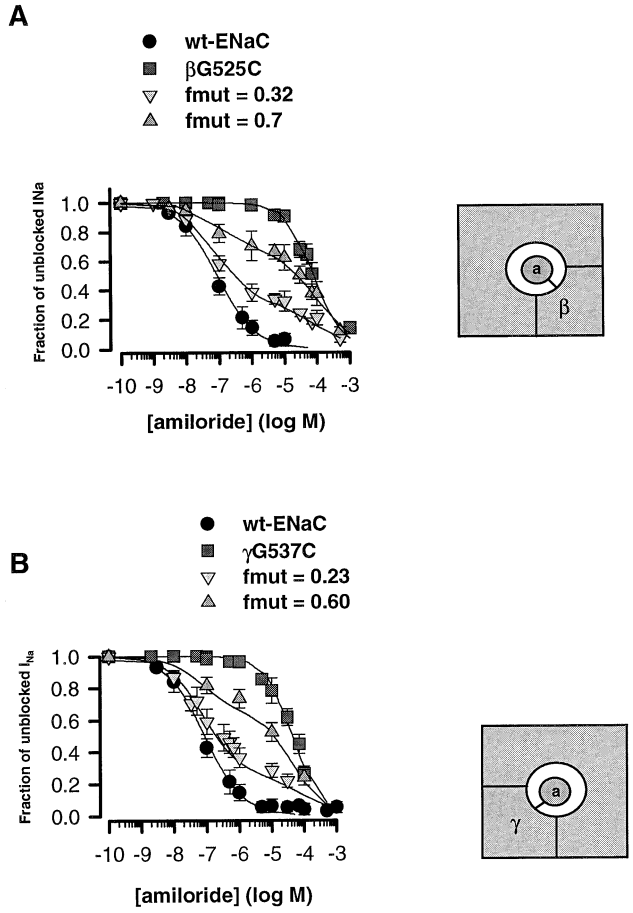

Fig. 3. Amiloride inhibition curves of macroscopic Na^+ current (I_{Na}) expressed by heteromultimeric αβγ ENaC made of wild-type and/or βγ mutant subunits. (**A**) Wild-type αβγ ENaC (●) shows a K_i for amiloride of 80 nM, and the $\alpha\beta_{G525C}\gamma$ mutant (■) a K_i of 87 μM according to best fit of the data to the Langmuir isotherm. Amiloride inhibition curves obtained from mixtures of the β_{G525C} mutant and the wild-type β subunit at different fractions of mutant subunit [f_{mut} of 0.32 (▽) and 0.7 (△)] are biphasic. Data (▽,△) were fitted (solid lines) to the equation in Figure 2 legend, assuming a K_i for amiloride of 80 nM for the sensitive component and 87 μM for the resistant component of I_{Na}, and two distinct channel species (wild-type or mutant) with one β subunit. The schematic illustration represents one β subunit contributing to channel block by amiloride. (**B**) Low amiloride affinity (K_i = 45 μM) of the γ_{G537C} mutant (■). Data from fractional expression of the γ_{G537C} mutant (f_{mut}) of 0.60 (△) and 0.23 (▽) over the wild-type γ subunit were fitted (solid lines) to the equation in Figure 2 legend, with a K_i for amiloride block of 80 nM and 45 μM for the amiloride-insensitive and -resistant components of the titration curve. Fit assumes two channel species with a single γ subunit involved in block by amiloride as shown in the schematic illustration of the ENaC channel.

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 β_{G525C} 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 γ_{G537C} mutant subunits exhibit a similar biphasic shape to that observed for the β_{G525C} 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 γ_{G537C} 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 γ_{G537C} 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 [^{35}S]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–α_{S583C} made from the α_{S583C} mutant subunit and wild-type β and γ subunits, generated a robust amiloride-sensitive Na^+ current. The current expressed was sensitive to Zn^{2+} block, indicating that the α_{S583C} subunit of the trimer participates in the formation of the channel pore (Figure 5A). When the β–γ–α_{S583C} trimer was co-expressed with wild-type α subunits, 50% of the current was resistant 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+}

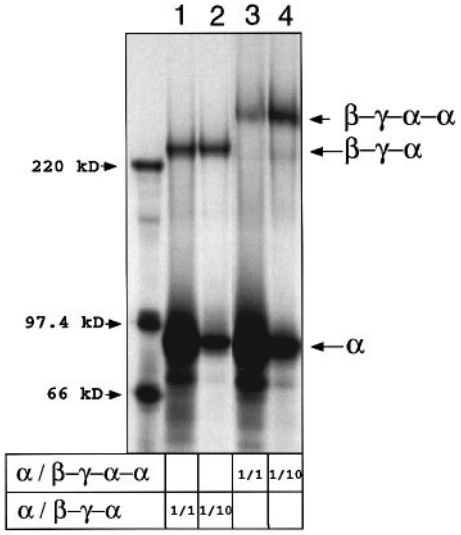

Fig. 4. Immunoprecipitation profile of co-expressed β–γ–α or β–γ–α–α cDNA constructs together with the α ENaC subunit. Oocytes were injected with β–γ–α or β–γ–α–α cRNAs with α cRNA in a molar ratio of either 1:1 or 10:1 respectively

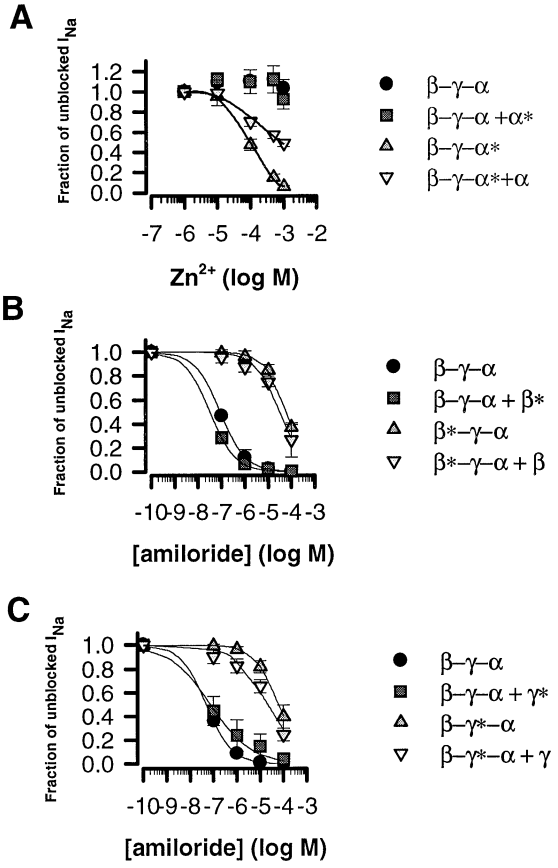

Fig. 5. Complementation of tandem trimeric β–γ–α constructs by monomeric α, β or γ subunits. (**A**) Zn^{2+} titration curve of Na^+ current (I_{Na}) expressed by the wild-type trimeric construct β–γ–α alone (●, mean I_{Na} = 32 ± 12 μA) or co-expressed with the α_{S583C} subunit (■, mean I_{Na} 15 ± 6 μA), and the β–γ–α_{S583C} trimer (▲, mean I_{Na} = 28 ± 12 μA) alone or with α wild-type subunit (▲, mean I_{Na} = 15 ± 9 μA). Fit of the data gave a K_i of 100 μM for Zn^{2+} block of the β–γ–α_{S583C}. In oocytes expressing β–γ–α_{S583C} together with the α wild-type subunit, 50% of I_{Na} is insensitive to Zn^{2+} block at a Zn^{2+} concentration of 1 mM. (**B**) Amiloride inhibition curve of I_{Na} expressed by the wild-type trimeric construct β–γ–α (●, mean I_{Na} = 6 ± 3.5 μA) alone or together with the β_{G525C} subunit (■, mean I_{Na} = 2.5 ± 1.6 μA) shows a K_i of 86 and 33 nM respectively. The amiloride K_i for the trimer β_{G525C}–γ–α (▲, mean I_{Na} = 5.2 ± 4.1 μA) was 58 and 31 μM when co-expressed with the wild-type β (▲, mean I_{Na} = 3.9 ± 2.9 μA). (**C**) Amiloride inhibition curve of I_{Na} expressed by the wild-type β–γ–α trimer (●, mean I_{Na} = 11 ± 9 μA, K_i = 49 nM) or co-injected with mutant γ_{G537C} subunits (■, mean I_{Na} = 6.2 ± 5 μA, K_i =75 nM). The trimeric mutant β–γ_{G537C}–α (▲, mean I_{Na} = 13 ± 6 μA) was resistent to amiloride (K_i = 62 μM) when expressed alone or together with the γ wild-type subunit (▲, mean I_{Na} = 8.6 ± 4 μA, K_i = 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, α_{S583C}, β_{G525C} or γ_{G537C}.

blockade. The interpretation of these results is illustrated in Figure 6A. When expressed alone, the β–γ–α_{S583C} trimer is complemented by a α_{S583C} subunit that is part of a separate polypeptide chain, and the two mutated α subunits confer Zn^{2+} sensitivity to the channel. When co-expressed with monomeric α subunits, complementation of the β–γ–α_{S583C} mutant trimer by a wild-type α subunit results in hybrid channels composed of one wild-type α and one α_{S583C} mutant subunit that are insensitive to Zn^{2+} in accordance with the dominant Zn^{2+}-resistant phenotype shown previously. The wild-type β–γ–α trimer is insensitive to Zn^{2+}, and co-expression with the α_{S583C} mutant subunit does not change channel resistance to Zn^{2+}, 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 α_{S583C} mutant subunit (data not shown). The evidence that the β–γ–α_{S583C} 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 Zn^{2+} 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 α_{S583C} mutants, this should have conferred, at least to some channels, the Zn^{2+}-sensitive phenotype that requires two cysteines on two α subunits. No Zn^{2+}-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 α_{S583C} mutant subunits, indicating that these trimers are indeed complemented by a single α_{S583C} 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 β_{G525C} or γ_{G537C} mutants did not significantly modify channel sensitivity to amiloride, indicating that monomeric β and γ mutants do not complement the trimer or compete with a homologous subunit to confer amiloride resistance to the channel (Figure 5B and C). As controls, trimeric constructs made with either β_{G525C} or γ_{G537C} mutant subunits expressed an amiloride-resistant I_{Na} confirming that both subunits participate in the formation of a functional channel. Co-expression of the mutant β_{G525C}–γ–α or β–γ_{G537C}–α trimeric construct with either the wild-type β or the γ subunit did not significantly change the channel resistance to amiloride. This lack of complementation of the β–γ–α trimer by β or γ subunits confirms that one β and one γ subunit are sufficient to form the functional channel protein. The

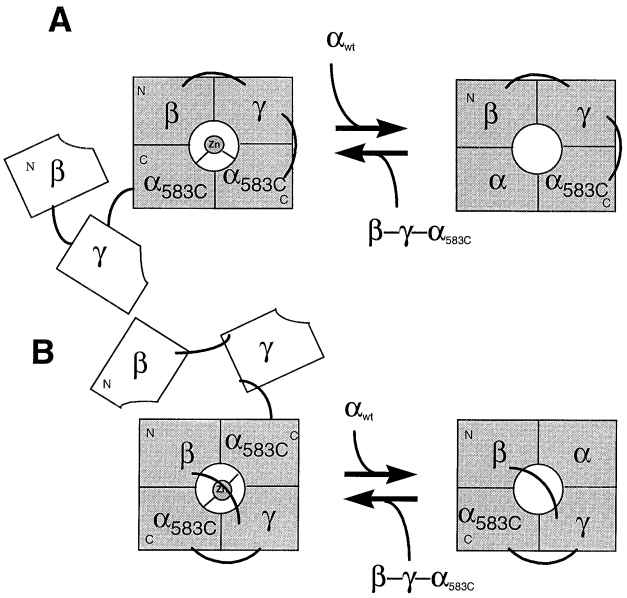

Fig. 6. Pictorial representation of possible subunit arrangements in a channel made of the mutant β–γ–α_{S583C} trimer complemented by a monomeric α wild-type subunit. When expressed alone, the β–γ–α_{S583C} trimer is complemented by the α subunit of another tandem trimer to form a functional channel. Co-expression of the β–γ–α trimer with α subunits leads to incorporation of a monomeric subunit resulting in two possible subunit arrangements. (**A**) The two neighbouring α subunits are flanked by β and γ subunits. (**B**) The two α subunits are separated by β and γ subunits. N and C denote the amino- and carboxy-termini of the polypeptide chain.

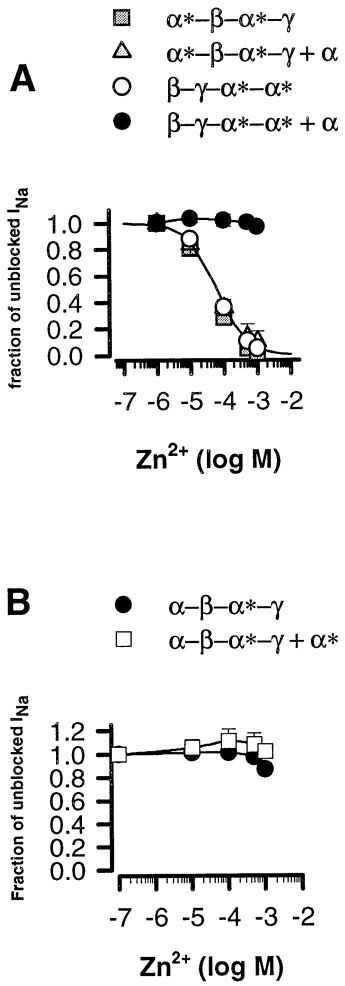

Fig. 7. Expression and Zn^{2+} sensitivity of $\alpha_2\beta_1\gamma_1$ tetrameric constructs. (**A**) Zn^{2+} titration curves of mutant β–γ–α_{S583C}–α_{S583C} (○, mean I_{Na} = 8.3 ± 6 μA) alone or co-expressed with the wild-type α subunit (●, mean I_{Na} = 21 ± 11 μA), and α_{S583C}–β–α_{S583C}–γ tetramers (▩, mean I_{Na} = 3.5 ± 3.3) alone or co-expressed with the wild-type α subunit (▲, mean I_{Na} = 3.8 ± 2 μA). The K_i of Zn^{2+} block was 43 μM for α_{S583C}–β–α_{S583C}–γ alone or co-expressed with monomeric α subunits, and 42 μM for β–γ–α_{S583C}–α_{S583C} tetramer expressed alone. *Denotes the α_{S583C} mutation. (**B**) Tetramers made of one mutated α_{S583C} are resistant to Zn^{2+} block (●, mean I_{Na} = 3 ± 1.9 μA), and co-expression with a monomeric α_{S583C} mutant (□, mean I_{Na} = 1.5 ± 0.9 μA) did not change the Zn^{2+}-resistant phenotype of the channel.

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 β–γ–α 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, α–β–α–γ and β–γ–α–α tetramers were constructed with the α_{S583C} mutation on both subunits. When expressed, both tetramers generated a robust amiloride-sensitive Na^+ current that was blocked by Zn^{2+} with a K_i of 42 μM (Figure 7A). Co-expression of the α_{S583C}–β–α_{S583C}–γ tetrameric construct with monomeric wild-type α subunits did not change the Zn^{2+} sensitity expressed by the tetramer alone. It indicates that the α_{S583C}–β–α_{S583C}–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 β–γ–α_{S583C}–α_{S583C} tetramer was complemented by a monomeric α subunit, as determined by a complete loss of channel sensitivity to Zn^{2+} block when the tetramer was co-expressed with α wild-type subunits. Complementation of the γ–α_{S583C}–α_{S583C}–β tetramer by α monomers could also be evidenced (data not shown). Thus only one α subunit of the β–γ–α–α 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 α_{S583C}–β–α_{S583C}–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 α_{S583C} and one wild-type α subunit is resistant to Zn^{2+} block, confirming our previous observation, made with co-expression of mixtures of α subunits, that Zn^{2+} blockade requires two mutated α_{583C} subunits. Co-expression of the α–β–α_{S583C}–γ tetramer with a mutated α_{S583C} subunit did not generate Zn^{2+}-sensitive channels, indicating that the α–β–α_{S583C}–γ tetramer is not complemented by the monomeric α_{S583C} subunit. Finally, we have verified at the single

channel level that the α–β–α–γ 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 α, β and γ subunits that participate in the formation of the ion-conducting structure of the channel. A quantitative analysis of cell surface expression of ENaC α, β and γ subunits enabled us to show that ENaC subunits assemble according to a fixed stoichiometry, and that the number of α subunits normalized per functional channel expressed at the cell surface is higher than β or γ 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 α, one β and one γ subunit that assemble pseudosymetrically 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 α subunits separated by one β and one γ subunit.

In the *Xenopus* oocyte expression system, the α, β and γ subunits are necessary for maximal expression of functional channel complexes. Previous reports have shown that individual β or γ 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 α subunits resulted in a small current amplitude that represents at most 1% of the amiloride-sensitive current resulting from co-expression of α, β and γ subunits (Canessa *et al.*, 1993). This barely detectable amiloride-sensitive current resulting from expression of α subunits alone correlates with the low level of α subunits present at the cell surface as if α subunits were not efficiently targeted to the plasma membrane in the absence of β and γ 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 α together with β or α with γ subunits, generates functional amiloride-sensitive channels with slightly different functional properties (McNicholas and Canessa, 1997). Once again, the level of expression of these αβ or αγ 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 α, β and γ subunits. When the α, β and γ subunits are co-expressed, we have been able to show that the subunits in the αβγ channel complex are not interchangeable, implying a preferential assembly that involves α, β and γ subunits. The preferred αβγ 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 α, β and γ 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 α-β–α–γ 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 α_1, β_1 and γ_2 subunits, assembly results in a preferred $\alpha_1\beta_1\gamma_2$ form of the channel and the functional $\alpha_1\beta_1$ channel 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 α subunits, leading to $\alpha_2\beta_2$ 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 α 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 α subunit and the corresponding Gly525 and Gly537 residues on the β and γ 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 αSer_{583}, βGly_{525} and γGly_{537} may well represent a similar hairpin structure preceding a transmembrane α helix involved in the outer mouth of the channel pore. Consistent with this model, experiments using proteolytic digestion of αENaC have demonstrated that a short segment encompassing Ser583 in the α 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}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 α, β and γ 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 α, β and γ subunit were synthesized *in vitro* and stored at a concentration of 100 ng/μl. Oocytes were injected with 3.3 ng of cRNA encoding each α, β and γ 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 total cRNA) able to produce maximal expression of ENaC channels. Amiloride-sensitive Na^+ current (I_{Na}) was recorded with a two-microelectrode voltage clamp at a holding potential of –100 mV as previously described (Schild *et al.*, 1995).

In oocytes expressing a mixture of blocker-sensitive and -insensitive channels, the overall unblocked fraction (U_{mix}) at a defined blocker concentration ([B]) is equal to the sum of the unblocked fractions due to each channel species (*i*) (MacKinnon, 1991):

$$U_{\mathrm{mix}} = \sum_{i=0}^{n} F_{\mathrm{i}} \left(\frac{K_{\mathrm{i}}}{K_{\mathrm{i}} + [\mathrm{B}]} \right) \quad (1)$$

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

$$F_{\mathrm{i}} = \binom{n}{i} f_{\mathrm{mut}}^{\,i} * f_{\mathrm{wt}}^{\,(n-i)} \quad (2)$$

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

The fractions f_{wt} and f_{mut} expressed in oocytes were determined by known ratios of cRNAs assuming that wild-type and mutant subunits are expressed to the same extent and assemble in a random fashion. I_{Na} expressed by the $\alpha_{S583C}\beta\gamma$, $\alpha\beta_{G525C}\gamma$ and $\alpha\beta\gamma_{G537C}$ 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 $\alpha_{S583C}\beta\gamma$ mutant and α wild-type, the fractional expression of the mutant channel types is lower than expected from the amount of cRNA injected, and f_{mut} becomes 0.43 (0.76:1) instead of 0.5 (1:1). Similarly, a 1:1 cRNA mixture of $\alpha\beta_{G525C}\gamma$ mutant and β wild-type effectively corresponds to a f_{mut} value of 0.32, and a f_{mut} of 0.60 for a 1:1 cRNA mixture of $\alpha\beta\gamma_{G537C}$ mutant over γ wild-type. The condition of random assembly of wild-type and mutant subunits to form functional channels was verified by comparison of binding of [^{125}I]anti-FLAG mAb to FLAG-tagged mutant subunits and the FLAG tagged wild-type subunit when co-expressed with identical concentration of cRNAs. Paired experiments gave similar cell surface expression of mutant and wild-type subunits for 1:1 mixtures of α_{S583C}:α wild-type, β_{G525C}:β wild-type and γ_{G537C}:γ 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 *Hpa*I restriction endonuclease coding sequence (GTT/AAC) followed by a unique *Xho*I 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 *Eco*RV 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 *Eco*RV and *Xho*I and insertion of the digestion product into the '3′-modified construct' cut with *Hpa*I–*Xho*I.

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 trimer or tetramer and 1 ng of cRNA encoding the monomeric subunit to obtain equal amounts of synthesized protein.

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References

Angelotti,T.P. and Macdonald,R.L. (1993) Assembly of $GABA_A$ receptor subunits: $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_2$ subunits produce unique ion channels with dissimilar single-channel properties. *J. Neurosci.*, **13**, 1429–1440.

Canessa,C.M., Horisberger,J.-D. and Rossier,B.C. (1993) Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature*, **361**, 467–470.

Canessa,C.M., Merillat,A.-M. and Rossier,B.C. (1994a) Membrane topology of the epithelial sodium channel in intact cells. *Am. J. Physiol. Cell Physiol.*, **267**, C1682–C1690.

Canessa,C.M., Schild,L., Buell,G., Thorens,B., Gautschi,I., Horisberger,J.-D. and Rossier,B.C. (1994b) Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature*, **367**, 463–467.

Chang,S.S. *et al*. (1996). Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1. *Nature Genet.*, **12**, 248–253.

Du,H.P., Gu,G.Q., William,C.M. and Chalfie,M. (1996) Extracellular proteins needed for *C.elegans* mechanosensation. *Neuron*, **16**, 183–194.

Farman,N., Talbot,C.R., Boucher,R., Fay,M., Canessa,C., Rossier,B. and Bonvalet,J.P. (1997) Noncoordinated expression of α, β, and γ subunit mRNAs of epithelial Na^+ channel along rat respiratory tract. *Am. J. Physiol. Cell Physiol.*, **41**, C131–C141.

Firsov,D., Schild,L., Gautschi,I., Mérillat,A.-M., Schneeberger,E. and Rossier,B.C. (1996) Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. *Proc. Natl Acad. Sci. USA*, **93**, 15370–15375.

Garcia-Anoveros,J., Derfler,B., Nevillegolden,J., Hyman,B.T. and Corey,D.P. (1997) BNaC1 and BNaC2 constitute a new family of human neuronal sodium channels related to degenerins and epithelial sodium channels. *Proc. Natl Acad. Sci. USA*, **94**, 1459–1464.

Huang,M. and Chalfie,M. (1994) Gene interactions affecting mechanosensory transduction in *Caenorrhabditis elegans*. *Nature*, **367**, 467–470.

Lai,C.C., Hong,K., Kinnell,M., Chalfie,M. and Driscoll,M. (1996) Sequence and transmembrane topology of MEC-4, an ion channel subunit required for mechanotransduction in *Caenorhabditis elegans*. *J. Cell Biol.*, **133**, 1071–1081.

Liman,E.R., Tytgat,J. and Hess,P. (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron*, **9**, 861–871.

Lingueglia,E., Champigny,G., Lazdunski,M. and Barbry,P. (1995) Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. *Nature*, **378**, 730–733.

MacKinnon,R. (1991) Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature*, **350**, 232–235.

McNicholas,C.M. and Canessa,C.M. (1997) Diversity of channels

generated by different combinations of epithelial sodium channel subunits. *J. Gen. Physiol*., **109**, 681–692.
Renard,S., Lingueglia,E., Voilley,N., Lazdunski,M. and Barbry,P. (1994) Biochemical analysis of the membrane topology of the amiloride-sensitive Na^+ channel. *J. Biol. Chem*., **269**, 12981–12986.
Schild,L., Canessa,C.M., Shimkets,R.A., Gautschi,I., Lifton,R.P. and Rossier,B.C. (1995) A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. *Proc. Natl Acad. Sci. USA*, **92**, 5699–5703.
Schild,L., Schneeberger,E., Gautschi,I. and Firsov,D. (1997) Identification of amino acid residues in the α, β, γ subunits of the epithelial sodium channel (ENaC) involved in amiloride block and ion permeation. *J. Gen. Physiol*., **109**, 15–26.
Shimkets,R.A. *et al*. (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the β subunit of the epithelial sodium channel. *Cell*, **79**, 407–414.
Snyder,P.M., McDonald,F.J., Stokes,J.B. and Welsh,M.J. (1994) Membrane topology of the amiloride-sensitive epithelial sodium channel. *J. Biol. Chem*., **269**, 24379–24383.
Tavernarakis,N. and Driscoll,M. (1997) Molecular modeling of mechanotransduction in the nematode *Caenorhabditis elegans*. *Annu. Rev. Physiol*., **59**, 659–689.
Waldmann,R., Champigny,G., Bassilana,F., Heurteaux,C. and Lazdunski,M. (1997) A proton-gated cation channel involved in acid-sensing. *Nature*, **386**, 173–177.

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