Subunit Stoichiometry of a Core Conduction Element in a Cloned Epithelial Amiloride-Sensitive Na^+ Channel

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ABSTRACT The molecular composition of a core conduction element formed by the α-subunit of cloned epithelial Na^+ channels (ENaC) was studied in planar lipid bilayers. Two pairs of in vitro translated proteins were employed in combinatorial experiments: 1) wild-type (WT) and an N-terminally truncated $\alpha_{\Delta N}$-rENaC that displays accelerated kinetics ($\tau_o = 32 \pm 13$ ms, $\tau_c = 42 \pm 11$ ms), as compared with the WT channel ($\tau_{c1} = 18 \pm 8$ ms, $\tau_{c2} = 252 \pm 31$ ms, and $\tau_o = 157 \pm 43$ ms); and 2) WT and an amiloride binding mutant, $\alpha_{\Delta 278-283}$-rENaC. The channels that formed in a α_{WT}:$\alpha_{\Delta N}$ mixture fell into two groups: one with τ_o and τ_c that corresponded to those exhibited by the $\alpha_{\Delta N}$-rENaC alone, and another with a double-exponentially distributed closed time and a single-exponentially distributed open time that corresponded to the α_{WT}-rENaC alone. Five channel subtypes with distinct sensitivities to amiloride were found in a $1\alpha_{WT}$:$1\alpha_{\Delta 278-283}$ protein mixture. Statistical analyses of the distributions of channel phenotypes observed for either set of the WT:mutant combinations suggest a tetrameric organization of α-subunits as a minimal model for the core conduction element in ENaCs.

INTRODUCTION

The amiloride-sensitive conductance pathway in Na^+-reabsorbing epithelia has been attributed to three cloned homologous subunits, termed α-, β-, and γ-ENaC (for epithelial Na^+ channel) (Canessa et al., 1993, 1994; Linguegliа et al., 1993). α-ENaC by itself (but not β- or γ-) is sufficient to form functional amiloride-sensitive Na^+ channels in lipid bilayers and in *Xenopus* oocytes (Canessa et al., 1993; Ismailov et al., 1996). The role of β- and γ-subunits in channel function remains largely unknown, although the presence of these subunits increases amiloride-sensitive current (I_{amil}) and contributes to amiloride sensitivity of this current in oocytes (Canessa et al., 1994; McNicholas and Canessa, 1997; Schild et al., 1997). These findings formed the basis for the hypothesis that a core conduction element in ENaCs can be formed by α-subunit(s) alone. The present study was performed to determine the molecular architecture of this core conduction element, in particular, the stoichiometry of the α-subunits in a homo(multi)meric ENaC.

The stoichiometry of ion channels has been successfully studied by using a combinatorial approach (MacKinnon, 1991). In its original design, the method involves the electrophysiological analyses of cells expressing RNA encoding wild-type (WT) and mutant channels in different proportions, and the solution of the problem is minimized to a set of derivations in terms of combinatorics. The present study applies this approach to single-channel experiments in planar lipid bilayers—a system that allows a high level of control over the ratio of subunits forming channels. It is based on the previous finding that in vitro translated α-ENaC subunits self-assemble into functional channels in liposomes and can be reconstituted and studied in planar lipid bilayers (Ismailov et al., 1996, 1997c). The studies of the stoichiometry of the core conduction element of ENaC were performed in this cell free system, using mixtures of the in vitro translated WT α-rENaC with either the N-terminally truncated gating mutant $\alpha_{\Delta N}$-rENaC or with the amiloride-binding mutant $\alpha_{\Delta 278-283}$-rENaC. The channels found in α_{WT}:$\alpha_{\Delta N}$ mixtures formed two groups: one with τ_o and τ_c, which correspond to the N-terminally truncated channels, and another with τ_o and τ_c, which correspond to the WT α-rENaC. Five distinct functional phenotypes formed by the α_{WT}:$\alpha_{\Delta 278-283}$-rENaC mixtures were distributed binomially within a range of two orders of magnitude of amiloride sensitivities. Statistical analyses of channel phenotypes in both sets of experiments suggest a tetrameric organization of α-subunits as a minimal model for the core conduction element.

MATERIALS AND METHODS

Reagents

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals were reagent grade, and all solutions were made with distilled water and filter sterilized before use (Sterivex-GS, 0.22 μm filter; Millipore Corp., Bedford, MA).

Preparation of α-rENaC constructs

The amiloride-binding domain mutant $\alpha_{\Delta 278-283}$-rENaC was generated as described previously (Ismailov et al., 1997b). N-terminally truncated $\alpha_{\Delta N}$-

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rENaC was constructed in two steps. A 250-bp polymerase chain reaction (PCR) fragment was synthesized using α-rENaC DNA as a template, with a sense primer (CGACGTCGACCATGGCCTTCTGGGCGGTGCTGT) corresponding to nucleotides 328–347 of α-rENaC and an antisense primer (TTGTACAAGTCAAAAAGCGTCTGCTCCGTG) corresponding to nucleotides 534–564. The sense primer also contained an upstream *Sal*I restriction site, a partial Kozak consensus site sequence, and an initiator methionine codon, and the antisense primer included a BsrGI restriction site. The 250-bp PCR product was subcloned into pcR 2.1 (TA Cloning Kit, In Vitrogen), and the sequence was verified by ABI PRISM dye terminator cycle sequencing (Perkin-Elmer). α-rENaC and the subcloned PCR product were digested with *Sal*I/BsrGI, and the gel-purified PCR fragment was ligated into digested pSport/α-rENaC vector. The sequences of both strands were confirmed by ABI PRISM dye terminator cycle sequencing. Thus, in $\alpha_{\Delta N}$-rENaC the N-terminal 108 amino acids have been deleted, and Tyr^{109} has been replaced by a methionine. Capped cRNA was prepared according to the manufacturer's protocol (mMessage Machine kit, Ambion) from Not I linearized vector, using T7 RNA polymerase.

In vitro translation and reconstitution into proteoliposomes

Individual α-rENaC proteins were in vitro translated with a TNT transcription-translation kit (Promega, Madison, WI), according to the manufacturer's instructions, in the presence of canine microsomal membranes (Promega) and 0.8 mCi/ml [^{35}S]translabel methionine (ICN, Costa Mesa, CA). A 25-μl translation reaction for each DNA was mixed with 0.5 mg phosphatidylethanolamine, 0.3 mg phosphatidylserine, 0.2 mg phosphatidylcholine, Triton X-100 (0.2% (v/v) final concentration), and 25 μl of a buffer containing 60 mM Tris(hydroxymethyl)aminomethane (Tris) (pH 6.8), and 25% glycerol (v/v). The translated proteins were eluted from a G-150 superfine Sephadex (Pharmacia Biotech.) gel filtration column with a buffer containing 500 mM NaCl, 1 mM EDTA, and 10 mM Tris (pH 7.6), and reconstituted into proteoliposomes as described previously (Ismailov et al., 1996, 1997c) at a given molar ratio based on the [^{35}S]methionine/cysteine labeling for each subunit. Mock controls were prepared by performing the in vitro translation reaction in the absence of rENaC cRNA and reconstituting purified reaction products into proteoliposomes according to an identical protocol.

Proteoliposomes containing in vitro translated WT and mutant α-rENaC proteins were sonicated (43 kHz, 160 W, 40 s) in the presence of 50 μM dithiothreitol (DTT) and allowed to reform by freeze-thawing three to five times. This protocol was developed to dissociate putative individual conduction elements of ENaC held together by sulfhydryl bonds (Ismailov et al., 1996). Our experiments demonstrate that with this procedure, single channels with uniform conductance of 13 pS were observed in over 70% of total incorporations. If multiple channel incorporations occurred, the sonication-freeze/thawing procedure was repeated. No differences in unitary conductance, ion selectivity, or amiloride sensitivity of single channels formed by the WT or mutant α-rENaC pretreated with 50 μM DTT were found, as compared with the nontreated channels. DTT at concentrations above 50 μM led to irreversible loss of ENaC activity (Ismailov et al., 1996).

Planar lipid bilayer experiments

Mueller-Rudin planar lipid bilayers with a membrane capacitance of 200–300 pF (0.67–0.95 μF/cm^2) were routinely formed from a 2:1 (w/w) diphytanoylphosphatidylethanolamine/diphytanoylphosphatidylserine membrane-forming solution in *n*-octane (final lipid concentration 25 mg/ml). The bilayers were bathed with symmetrical 100 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid-Tris (MOPS-Tris) buffer (pH 7.4). Single-channel currents were measured as described previously (Ismailov et al., 1996, 1997c), using a conventional current-to-voltage converter with a 1-GΩ feedback resistor (Eltec, Daytona Beach, FL) connected to the *cis* compartment, and the *trans* compartment clamped at 100 mV. Single-channel incorporations were selected for further experimentation by activating all incorporated channels with the imposition of a hydrostatic pressure gradient, as described previously (Ismailov et al., 1997a). Single-channel analyses were performed with pCLAMP 5.6 software (Axon Instruments, Burlingame, CA) on current records low-pass filtered at 300 Hz through an 8-pole Bessel filter (902 LPF; Frequency Devices, Haverhill, MA) before acquisition with a Digidata 1200 interface (Axon Instruments). For illustration purposes, records were filtered at 100 Hz, using a built-in digital filtering option of the pCLAMP 5.6 software (Axon Instruments).

Combinatorial experiments

The combinatorial approach to determining the subunit stoichiometry of ion channels (MacKinnon, 1991) is based on derivations from probability laws. The probabilities of random association of two elements into an *N*-oligomer with *m* subunits substituted by a mutant (P_m^N, where *m* varies from zero to *N*) are determined as

$$P_m^N = F_{WT}^{N-m} \cdot F_{mut}^{m} \cdot C(N, m) \quad (1)$$

where F_{WT} and F_{mut} are the relative molar fractions of the WT and mutant channel subunits; and the binomial coefficient $C(N, m)$ stands for the number of all possible combinations of WT and mutant subunits:

$$C(N, m) = N!/(m! \cdot (N - m)!) \quad (2)$$

A basic property of the binomial series is that the sum of these probabilities is equal to unity:

$$\sum P_m^N = \sum (F_{WT}^{N-m} \cdot F_{mut}^{m} \cdot C(N, m)) = 1 \quad (3)$$

and the total number of combinations of two (WT and mutant) elements within a given *N*-element set (*N*-oligomer) is

$$\sum C(N, m) = 2^N \quad (4)$$

This number of combinations of WT and mutant subunits does not necessarily equal the number of channel phenotypes; however, this possibility cannot be excluded a priori. Combinatorial experiments at the single-channel level eliminate the uncertainties in interpretation when multiple channels are examined, including the variety of hybrid channels, and allow direct analyses of statistical distributions of channels formed by different mixtures of the WT and mutant proteins.

RESULTS

The stoichiometry of α-ENaC was initially assessed using combinations of WT and $\alpha_{\Delta N}$-rENaC. These proteins form channels with very different kinetic properties (Fig. 1). Dwell-time histogram analysis of single-channel data revealed that the time spent by WT α-rENaC in its closed state is distributed double exponentially ($\tau_{c1} = 18 \pm 8$ ms, $\tau_{c2} = 252 \pm 31$ ms), whereas the closed time of $\alpha_{\Delta N}$-rENaC could be fit to a single exponential ($\tau_c = 42 \pm 11$ ms). The open time histograms for both channels could be described as single exponentials, but with quite different mean open times ($\tau_o = 157 \pm 43$ ms in the WT channel and $\tau_o = 32 \pm 13$ ms in the mutant). These differences in kinetic properties are consistent with the hypothesis that the N-terminus of α-ENaC participates in channel gating (Grunder et al., 1997) and were used as quantitative parameters in our combinatorial experiments.

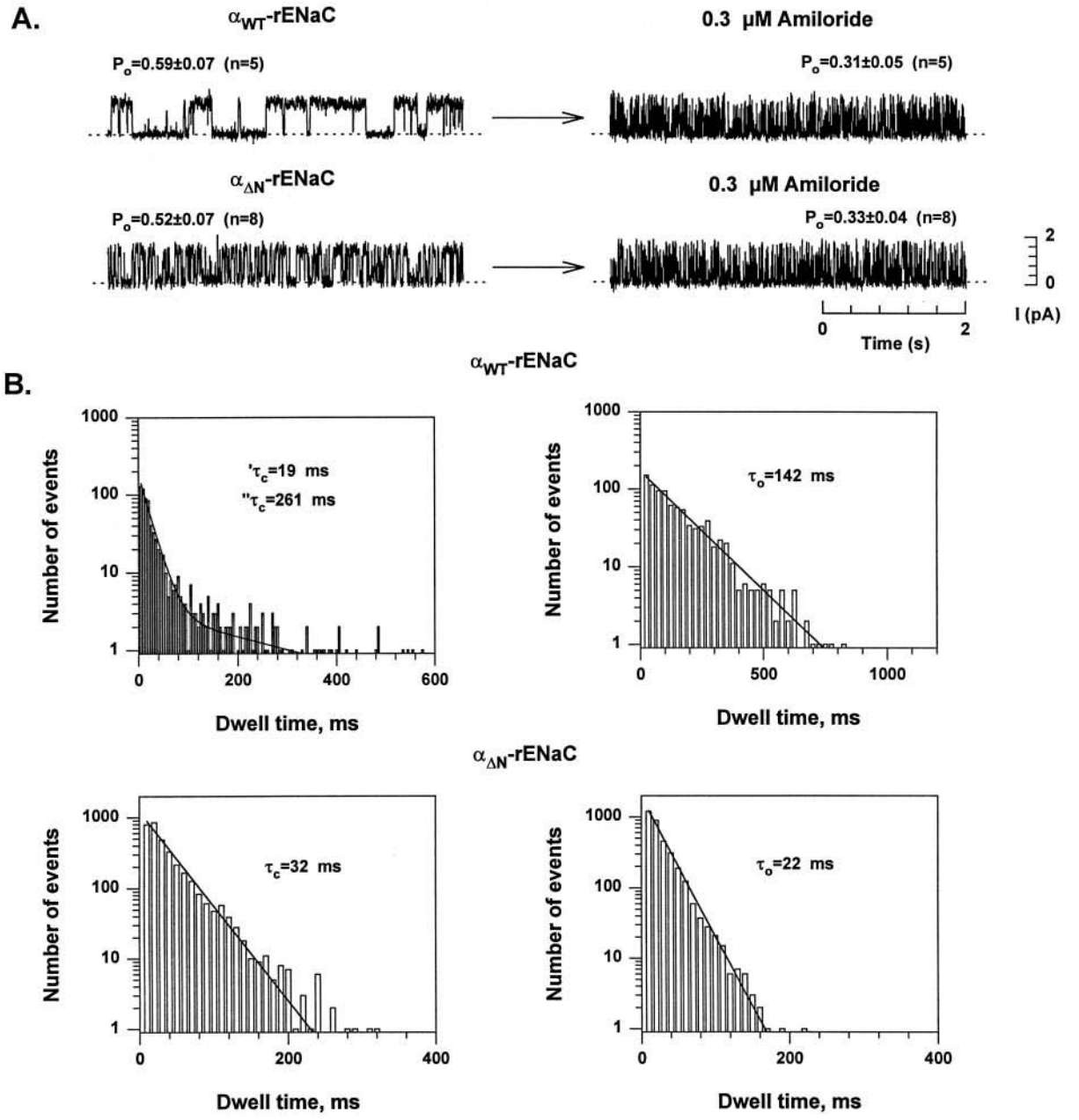


FIGURE 1 Kinetic properties of the WT and the N-terminally truncated α-rENaC. Proteoliposomes containing mixtures of in vitro translated WT or N-terminally truncated α-rENaC were formed in the presence of 50 μM DTT. Bilayers were bathed with symmetrical 100 mM NaCl solution complemented with 10 mM Tris-MOPS (pH 7.4). The holding potential was +100 mV. For illustration purposes, records (*A*) were filtered at 100 Hz and are representative of at least five separate experiments with each channel. Amiloride was added to the *trans* compartment of the bilayer chamber at 0.3 μM to ascertain the identity of channels. Representative dwell-time histograms of α_{WT}-rENaC and $\alpha_{\Delta N}$-rENaC (*B*) were constructed after event analysis with pCLAMP software (Axon Instruments) on single-channel recordings of 3 min duration, filtered at 300 Hz with an 8-pole Bessel filter before acquisition at 1 ms per point with pCLAMP software and hardware. Event detection thresholds were 50% in amplitude of transition between closed and open states, and 3 ms for event duration. Numbers of events used for histogram construction were 888 and 3355 for α_{WT}-rENaC and $\alpha_{\Delta N}$-rENaC, respectively. The bin widths in the closed and open time histograms for α_{WT}-rENaC were 5 and 25 ms, respectively. Closed and open time constants were determined from double-exponential ($y = a_1 \cdot \exp(-x/\tau_1) + a_2 \cdot \exp(-x/\tau_2)$) and single-exponential ($y = a \cdot \exp(-x/\tau)$) fits of the closed and open time histograms, respectively. Closed and open time constants shown for $\alpha_{\Delta N}$-rENaC were determined from single-exponential fits of closed and open time histograms, respectively, binned with the 10-ms bin width.

Fig. 2 *A* depicts the typical results of combinatorial experiments performed with a mixture of the WT and $\alpha_{\Delta N}$-rENaC. In a 1:1 mixture, 39 cases of 42 total successful channel incorporations revealed the channels with open and closed times of 39 ± 11 ms and 35 ± 15 ms, respectively. These values correlate well with those observed for the N-terminally truncated mutant alone (Fig. 1); therefore, this type of channel was called type M′, for "mutant." The other

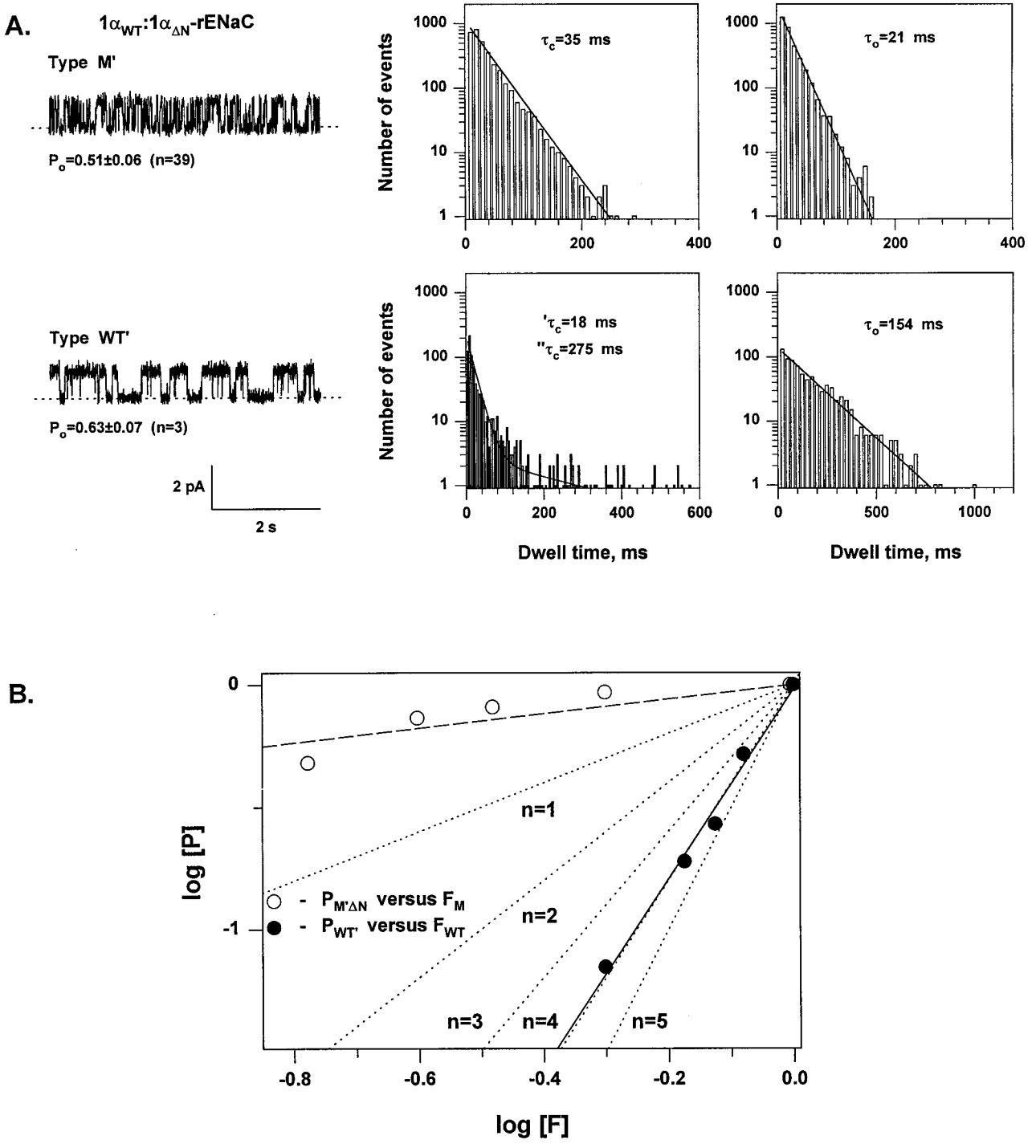


FIGURE 2 Kinetic properties and statistical distribution of the channels formed by different mixtures of WT and N-terminally truncated α-rENaC. Recording conditions and treatment of single-channel data were the same as described in Fig. 1. Single-channel recordings shown are representative of 39 separate observations for the type M′ and three separate observations for the type WT′ of 42 successful incorporations of the proteoliposomes containing a 1:1 mixture of in vitro translated N-terminally truncated and WT α-rENaCs (*A*). Distinction between channel types M′ and WT′ was made on the basis of open and closed time, as shown next to traces. Numbers of events used for construction of the histograms shown were 3328 (type M′) and 854 (type WT′). The kinetics of the channels found in all incorporations of the in vitro translated $\alpha_{WT}\alpha_{\Delta N}$-rENaCs mixed at different ratios were essentially undistinguishable from types M′ and WT′ shown, although they were distributed as shown in Table 1. The minimal number of separate observations that represent the fraction of channels in a double-logarithmic plot of statistical distribution of channel types (log[*P*]) at different α_{WT}:$\alpha_{\Delta N}$ ratios (log[*F*]) (*B*) was 3 for the type WT′ and 4 for the type M′ of the respective total number of successful incorporations of channels with a given composition (repeated with three different batches of proteoliposomes). The solid line represents a best first-order regression fit of the experimental data for the type WT′ (with a slope of 3.86). The dashed line represents a best first-order regression fit of the experimental data for the type M′ (with a slope of 0.30). Dotted lines represent theoretical probabilities of random association of two elements into a homo-N-oligomer when the ratio of these elements, calculated with Eqs. 5 and 6, is varied.

three successful incorporations resulted in channels with a double-exponentially distributed closed time (29 ± 13 ms and 243 ± 41 ms in duration) and a single-exponentially distributed open time (174 ± 33 ms). These kinetics resemble properties of the WT α-rENaC alone; therefore, these channels were called type WT′. The two phenotypes found in these experiments can be interpreted in combinatorial terms. If ENaC is made of more than one subunit, Eqs. 1–4 (see Materials and Methods) predict the existence of intermediate phenotypes due to the formation of hybrid channels. The absence of these intermediate phenotypes could mean that ENaC is a monomer or that WT and mutant proteins simply do not associate into a heteromultimeric channel. If this is the case, then the fractions of the WT and mutant channels should be directly proportional to the fractions of the WT and mutant proteins in the mixture; namely, at a 1:1 protein ratio the WT channels should be found as often as the mutant ones. Based on the observation that two types of channels were unequally distributed, we conclude that WT and mutant proteins do associate with each other to form functional channels that are composed of more than one α-rENaC subunit.

Furthermore, according to probability laws, the binomial coefficient for the homomultimeric combinations (fully WT or fully mutant) is equal to 1. This reduces Eq. 1 to

$$P_{WT} = F_{WT}^{N} \tag{5}$$

and

$$P_{M} = F_{mut}^{N} \tag{6}$$

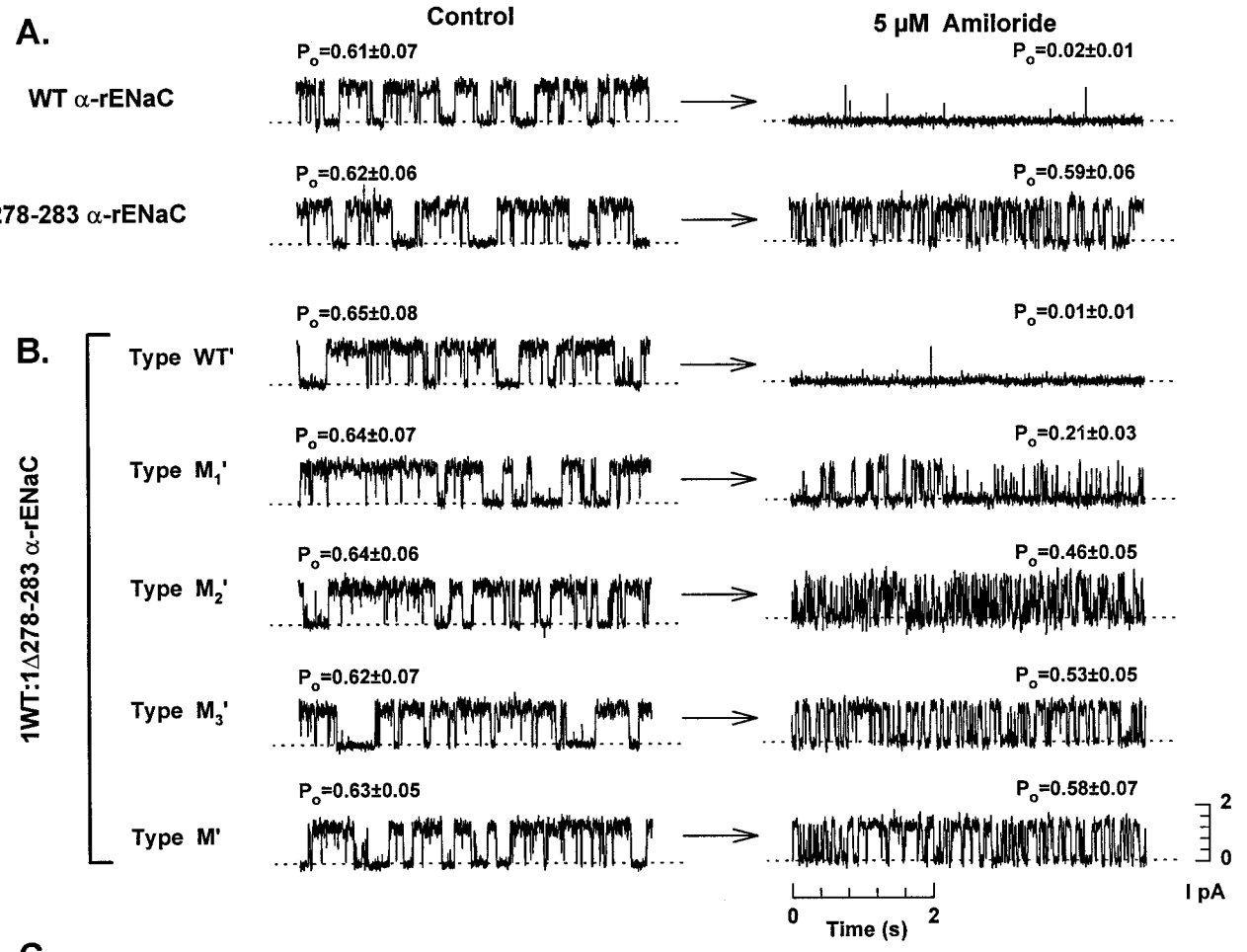
Accordingly, double-logarithmic plots of P versus F for fully WT or fully mutant channels over the range of the ratio of elements should be linear with slopes of N, where N is the number of subunits forming a multimer (see theoretical *dotted lines* in Fig. 2 *B* calculated for N from 1 to 5). To test these predictions, we next varied the ratio of the WT and mutant proteins in the proteoliposomes from 2:1 to 5:1 (WT:mutant). The fraction of channels with properties of the WT channel reconstituted from this mixture (WT′ type; *filled symbols* in Fig. 2 *B*; see also Table 1) plotted on a double-logarithmic scale versus the WT protein fraction in a mixture (where F_{WT} varies from 0.83 to 0.5) could be fit to a straight line with a slope of 3.86 (*solid line*). This slope suggests that four α-subunits form a functional channel. On the other hand, the fractions of channels with mutant properties reconstituted from these mixtures (M′ type; *open symbols* in Fig. 2 *B*; see Table 1 for numerical data) plotted on a double-logarithmic scale versus the respective fractions of mutant protein in a mixture (F_M varies from 0.17 to 0.5) were best fit with a slope of 0.3 (*dashed line*). This fit deviated from all predicted dependencies, including the unitary slope for a monomer, and may be explained as follows. Equation 4 predicts 16 different combinations of two (WT and mutant) elements for a tetramer. Two of these combinations should be homomultimeric (only WT, or only mutant), and 14 should be hybrids with one or as many as $m = N - 1$ WT element(s) substituted for a mutant subunit(s). These hybrid channels may be 1) channels that are not different from the WT; 2) channels that are not different from the mutant; or 3) channels that display graded changes in properties. Based on a relatively high proportion of the "mutant" phenotype in α_{WT}:$\alpha_{\Delta N}$-rENaC mixtures with no graded changes observed, we hypothesize that the N-termini of WT-subunits in all hybrid channels cannot override the defective gating that is the feature of the $\alpha_{\Delta N}$-mutant, i.e., the N-terminal deletion dominates the phenotype.

The above conclusion about the tetrameric organization of α-ENaC was based on the assumption that the change in gating results from substitution of a single WT subunit by a mutant subunit at the level of a monomer. However, if the substitution producing a change in channel function had occurred at the level of a dimer, the interpretation of combinatorial studies would be off by a factor of 2, and so on. To test for such a possibility, an independent estimate of α-rENaC stoichiometry was performed using a mixture of WT α-rENaC and another mutant, $\alpha_{\Delta 278-283}$-rENaC. This mutant has a greatly diminished (250-fold) sensitivity to amiloride as compared with the WT channel (Fig. 3 *A* and Ismailov et al., 1997b). The channels that were observed in combinatorial experiments using a mixture of the WT and $\alpha_{\Delta 278-283}$-rENaC had similar conductances, ion selectivity, and kinetics under control conditions, but differed in their sensitivities to amiloride. Five populations of channels with distinct sensitivities to amiloride were identified within a 1:1 (WT:mutant) protein mixture. Fig. 3 *B* depicts typical current records representative of each of these populations and the inhibition of these channels by amiloride. Amiloride dose-response curves were fit to the first-order Michaelis-Menten equation and are shown in Fig. 3 *C*. Although the inhibitions of the channels shown in Fig. 3, *B* and *C*, as types M_3' and M′ appear similar to each other, these channels form separate groups. This conclusion is made on the basis of the statistical distribution of the apparent inhibitory constants (K_i) determined for each channel found in a 1:1 mixture of the WT and the amiloride-binding domain mutant α-rENaC in 68 separate experiments (Fig. 3 *D*). The overall distribution of K_i values in this α_{WT}:$\alpha_{\Delta 278-283}$-rENaC mixture conforms to a binomial, as would be ex-

TABLE 1 Distribution of the single α-rENaCs in mixtures of the WT and N-terminally truncated mutant

WT:mutant ratio	n_{total}	$n_{WT'}$ ($P_{WT'}$)	$n_{M'}$ ($P_{M'}$)
5:1	25	13 (0.52)	12 (0.48)
3:1	15	4 (0.27)	11 (0.73)
2:1	21	4 (0.19)	17 (0.81)
1:1	42	3 (0.07)	39 (0.93)

$n_{WT'}$ represents the number of successful incorporations of channels with a double-exponentially distributed closed time (29 ± 13 ms and 243 ± 41 ms in duration) and a single-exponentially distributed open time 174 ± 33 ms (i.e., WT phenotype). $n_{M'}$ represents the number of incorporations of channels with τ_{open} = 39 ± 11 ms and τ_{closed} = 35 ± 15 ms (i.e., "mutant" phenotype). $P_{WT'}$ and $P_{M'}$ are the fractions of $n_{WT'}$ and $n_{M'}$ forming n_{total}.


A.
Control
5 μM Amiloride
WT α-rENaC
P_o=0.61±0.07
P_o=0.02±0.01
Δ278-283 α-rENaC
P_o=0.62±0.06
P_o=0.59±0.06
B.
1WT:1Δ278-283 α-rENaC
Type WT'
P_o=0.65±0.08
P_o=0.01±0.01
Type M1'
P_o=0.64±0.07
P_o=0.21±0.03
Type M2'
P_o=0.64±0.06
P_o=0.46±0.05
Type M3'
P_o=0.62±0.07
P_o=0.53±0.05
Type M'
P_o=0.63±0.05
P_o=0.58±0.07
2
0
I pA
0
Time (s)
2

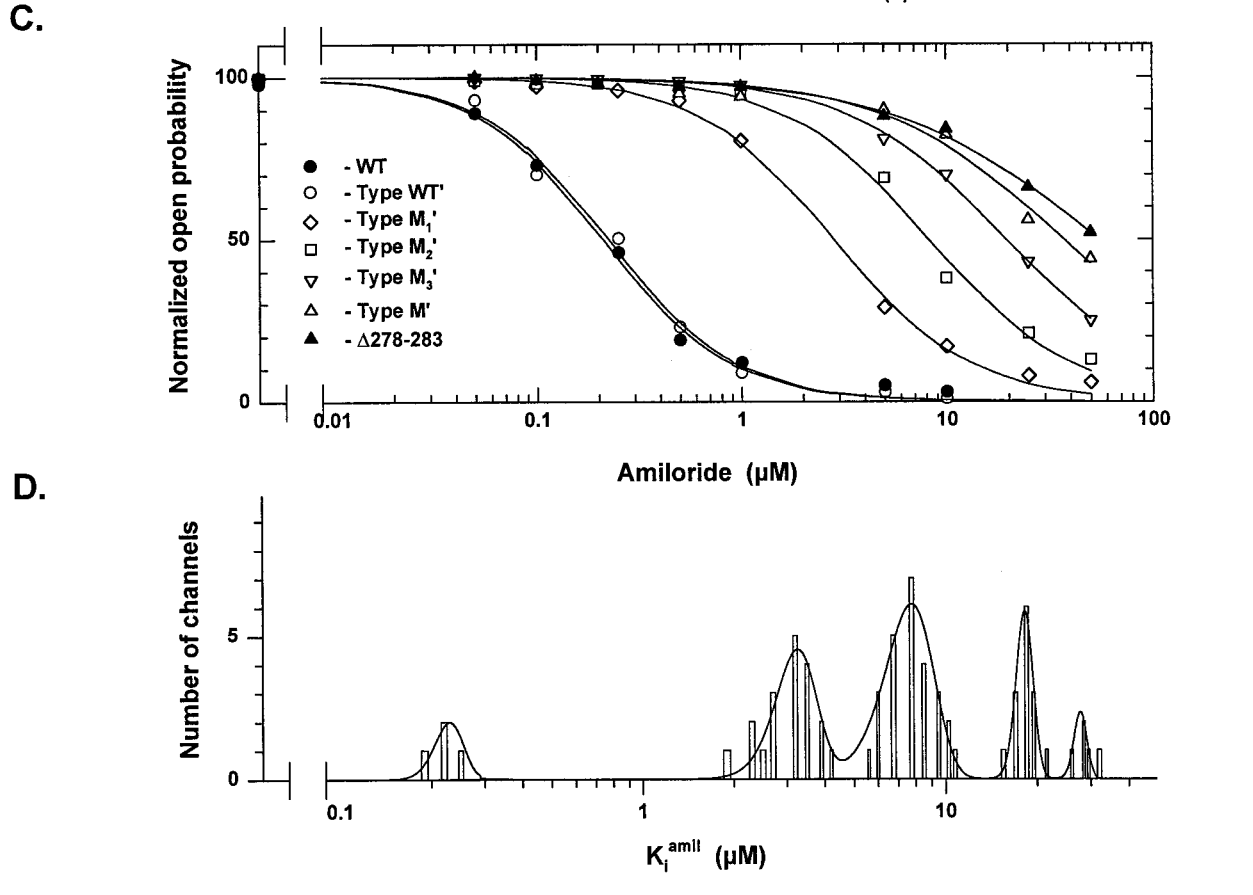

C.
Normalized open probability
100
50
0
- WT
- Type WT'
- Type M1'
- Type M2'
- Type M3'
- Type M'
- Δ278-283
0.01
0.1
1
10
100
Amiloride (μM)
D.
Number of channels
5
0
0.1
1
10
K_i^amil (μM)

TABLE 2 Distribution of the single α-rENaC in mixtures of the WT and amiloride-binding domain deletion mutant

K_i (10^{-6} M)		0.225 ± 0.035	3.25 ± 0.55	8.1 ± 3.2	18 ± 4	27 ± 4
WT:mutant ratio	n_{total}	$n_{WT'}$ ($P_{WT'}$)	$n_{M1'}$ ($P_{M1'}$)	$n_{M2'}$ ($P_{M2'}$)	$n_{M3'}$ ($P_{M3'}$)	$n_{M'}$ ($P_{M'}$)
5:1	27	12 (0.44)	11 (0.41)	4 (0.15)	(0.00)	(0.00)
2:1	29	6 (0.21)	11 (0.38)	9 (0.31)	3 (0.10)	(0.00)
1:1	68	4 (0.06)	19 (0.28)	26 (0.38)	14 (0.21)	5 (0.07)
1:2	33	−(0.00)	4 (0.12)	11 (0.33)	13 (0.39)	5 (0.15)

$n_{WT'}$ through $n_{M'}$ represent the number of successful incorporations of channels with the apparent inhibitory constant for amiloride K_i^{amil} indicated in the table, as determined in each particular channel by fitting the amiloride dose-response curves to the first-order Michaelis-Menten equation of n_{total} performed with a given mixture of the WT and $\alpha_{\Delta 278-283}$-rENaC. $P_{WT'}$ through $P_{M'}$ are respective fractions of WT′, M'_1, M'_2, M'_3, and M′ channels forming n_{total}.

pected for random combinations of two elements. Varying the protein ratio in the mixture shifts this distribution in such a way that the M′ fraction degenerates at WT:mutant ratios > 2:1, and the WT′ fraction decreases substantially at a WT:mutant ratio of 1:2 (Table 2). When P and F for the putative fully WT and fully mutant phenotype were plotted on a double-logarithmic scale (Fig. 4), both data sets could be fit to straight lines with nearly identical slopes of 3.89 (for M′) and 4.02 (for WT′). Moreover, these slopes are in agreement with the slope of 3.86 found for the WT′ fraction in the α_{WT}:$\alpha_{\Delta N}$-rENaC mixture (cf. Fig. 2 *B*). These data suggest that two populations of channels that exhibit WT and mutant phenotypes represent true homomultimers formed by the WT or amiloride-binding mutant α-rENaC, respectively. It is not entirely clear which of the aforementioned 14 possible hybrid combinations of WT and mutant subunits predicted from a binomial series for a tetramer can account for the three intermediate phenotypes found in the α_{WT}:$\alpha_{\Delta 278-283}$ mixture (M'_1 through M'_3 in Fig. 3). However, one can imagine a scenario in which the four singly substituted hybrids will be functionally indistinguishable from each other, as would be the six doubly substituted and the four triply substituted hybrids. Then a total of three functionally distinct hybrid phenotypes could be predicted.

In any case, our conclusion about the tetrameric organization of α-rENaC is based strictly on the statistical analyses of populations of channels that display the extreme (WT or mutant) phenotypes and does not involve the distribution of putative hybrids. Furthermore, this conclusion is strengthened by the fact that the distributions of these channels found in two independent series of combinatorial experiments using two different mutants can be fit to the function predicted from a binomial series for a tetramer.

DISCUSSION

The elementary conduction unit in ENaCs

To interpret the results of combinatorial experiments performed at the single-channel level, one must first define a *single channel*. Previously we reported that in vitro translated ENaCs form Na^+-selective channels in planar lipid bilayers and that these channels can be blocked by amiloride with a K_i of $\sim 10^{-7}$ M (Ismailov et al., 1996, 1997c). The gating of ENaC in bilayers does not appear to be independent, but rather concerted, with a 13-pS level that precedes the opening of the channel to a 39-pS conductance level (Ismailov et al., 1996, 1997a). This gating pattern can be disrupted using low concentrations ($\leq$50 μM) of the sulfhydryl-reducing agent DTT applied to the presumably extracellular surface. After DTT treatment, the channel conductance partitioned into three 13-pS conduction levels, and transitions between these levels became random. A triple-barrel model was developed to account for this behavior. This model describes ENaCs as formed by three individual conduction elements, each with a unitary conductance of 13 pS. Although this cooperative gating did not compromise in any way the initial studies of the biophysical properties of ENaC, it could confound the interpretation of combinatorial experiments based on a binomial formalism.

Therefore, the combinatorial experiments in the present study were performed in the channels pretreated with 50 μM DTT. The rationale for this maneuver was based on the consideration that if disulfide bonds hold together three individual but fully functional conduction elements, then by reducing these bonds before reconstitution, the individual conduction elements should distribute randomly between

FIGURE 3 Amiloride inhibition of single α-rENaC composed from the WT and amiloride-binding domain deletion mutant. The α_{WT}-rENaC and $\alpha_{\Delta 278-283}$-rENaC were in vitro translated individually, mixed at a 1:1 ratio, and reconstituted into proteoliposomes in the presence of 50 μM DTT. Recording conditions were as indicated in Fig. 1. (*A*) Typical records and effect of 0.3 μM of amiloride on channels formed by the α_{WT}-rENaC and $\alpha_{\Delta 278-283}$-rENaC alone (representative of at least five separate experiments with each channel). (*B*) Typical records of channels found in a 1:1 WT:mutant protein mixture and five typical responses of these channels to 0.3 μM amiloride: types WT′and M′ were undistingishable from the WT and mutant channels shown in *A*, and types M_1 through M_3 had intermediate sensitivities to amiloride. Complete amiloride dose-response curves, shown in *C* and determined for each population of channels shown in *B*, are representative of at least four experiments per group (see Table 2 for number of observations in each group). Solid lines in the amiloride dose-response graph represent best fits of the experimental data points to the first-order Michaelis-Menten equation rewritten as $P_o = P_o^{max} \cdot (1 - [\text{amiloride}]/K_i + [\text{amiloride}])$. The solid line in the statistical distribution of channel phenotypes bar graph (*D*) is a fifth-order Gaussian fit of the histogram.

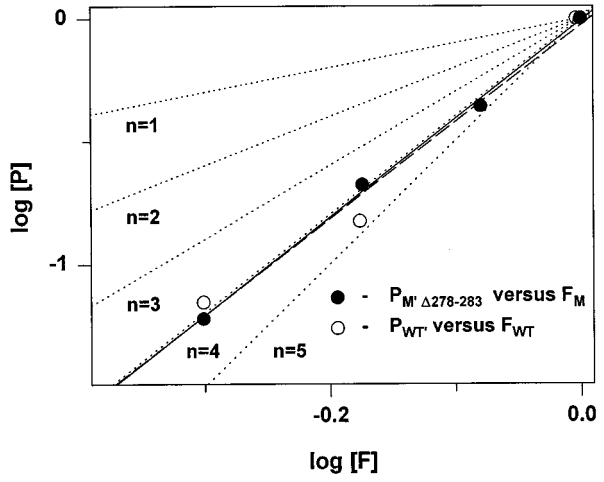


FIGURE 4 Statistical distribution of the single α-rENaC composed from the WT and amiloride-binding domain deletion mutant. Channel reconstitution, recording conditions, treatment of single-channel data, and distinction between channel types were the same as described in Fig. 3. The minimal number of separate observations that represent the fraction of channels in a double-logarithmic plot of statistical distribution of channel types at different α_{WT}:$\alpha_{\Delta 278-283}$ ratios was four for the type WT′ and five for the type M′, of the respective total number of successful incorporations of channels with a given composition (see Table 2 for the number of observations of each channel type at a given WT:mutant ratio). The solid line represents a best first-order regression fit of the experimental data for type M′ (with a slope of 3.89). The dashed line represents a best first-order regression of the experimental data for type WT′ (with a slope of 4.02). Dotted lines shown represent theoretical probabilities of random association of two elements into a homo-N-oligomer when the ratio of these elements, calculated with Eqs. 5 and 6, is varied.

proteoliposomes. This hypothesis was fully supported by the experimental data: the distribution of single-channel conductances after this procedure was perfectly uniform around 13 pS. Pretreatment with 50 μM DTT resulted in no other noticeable differences in ion selectivity, amiloride sensitivity, or the responses of single channels formed by the WT or mutant α-rENaC to actin. Based on these experiments, we conclude that DTT dissociates individual conduction elements in ENaC but does not otherwise affect the biophysical or pharmacological properties of these channels.

This definition of a single channel helps us to understand the first-order Michaelis-Menten kinetics of inhibition of these DTT-dissociated single channels with amiloride. In the experiments with "triple-barrel" channels reported previously (Ismailov et al., 1996, 1997b), a Hill coefficient greater than 1 was required to fit the data to Michaelis-Menten kinetics. The amiloride inhibition experiments reported here were performed on individual channels fully disassembled before reconstitution into planar lipid bilayers. Moreover, the amiloride inhibition constants and Hill coefficients found in combinatorial experiments performed on "triple-barrel" channels (i.e., channels not treated with DTT) were scattered over the entire range of amiloride sensitivities between fully WT and fully mutant channels with no apparent partititioning into distinct groups ($N = 72$; data not shown). This observation is consistent with the hypothesis that, with intact disulfide bonds, channels are assembled into a more complex agglomerate made of more than one conduction unit. Furthermore, we hypothesize that under these conditions the number of conduction units is three, which then would be consistent with the Hill coefficient of ~3 reported previously (Ismailov et al., 1996).

The distinct intermediate amiloride sensitivities that we observed in combinatorial experiments with disassembled individual conduction elements suggest that substitution of a single WT subunit for a mutant in the pore shifts the amiloride inhibition dose-response curve. One conclusion that follows from this observation is that each subunit interacts with amiloride independently of the others. The Hill coefficient for all hybrid channels as well as for the fully WT and fully mutant channels is close to unity. This implies that each of the four subunits that form the channel participate in the formation of a single amiloride-binding site. These findings should be compared with the two-binding-site "molecular plug" model for amiloride: one site within the electric field of the pore itself, presumably binding the guanidinium portion of molecule, and another site at the extracellular surface of the channel outside the pore "stabilizing" the block, presumably by binding the pyrazine portion of amiloride (for a discussion see Benos, 1982; Kleyman and Cragoe, 1988; Palmer, 1991). Combining this model with the proposed tetrameric organization of ENaCs, one can hypothesize that two different domains in each of the four ENaC subunits form these two binding sites, and these two sites interact with different portions of the same amiloride molecule in one kinetically indistinguishable step. Given the fact that amiloride is not a symmetrical molecule, it is unlikely that in a pseudosymmetrical tetrameric model of ENaC certain portions of amiloride (e.g., the Cl substituent at position 6 on the pyrazine ring, which is known to be important for binding and block; for a review, see Kleyman and Cragoe, 1988) will form covalent bond(s) with four binding sites in the channel at once. This may mean that other (noncovalent) types of interaction could take place between this molecule and multiple ENaC subunits, which is consistent with the reversibility of amiloride block of these channels. The domain in the extracellular loop of the channel protein mutated in these studies may represent the extracellular ("stabilizing") amiloride-binding site, whereas the second site remains intact in these mutants. A finite amiloride sensitivity of the fully mutant channel supports this conclusion.

Tetrameric organization of ENaCs as a minimal model

Comparison of the tetrameric model proposed here for α-rENaC with the stoichiometry of 2α:1β:1γ described recently for a heterooligomeric $\alpha\beta\gamma$-rENaC (Firsov et al., 1998; Kosari et al., 1998) suggests that the molecular organization of ENaCs parallels that of the inwardly rectifying voltage-insensitive K^+-selective channels. These K^+ chan-

nels are also made of four subunits and were shown to coassemble with auxiliary subunits and alternatively spliced variants, retaining the overall tetrameric organization (Duprat et al., 1995; Yang et al, 1995; Glowatzki et al., 1995; Wischmeyer et al., 1997; Woodward et al., 1997; Makhina and Nichols, 1998). This resemblance is intriguing, given the fact that these channels belong to different gene families and share no homology at the amino acid level. However, they have similar membrane topologies, namely, two α-helical membrane-spanning domains and a large extracellular loop (Snyder et al., 1994; Renard et al., 1994; Ho et al., 1993; Kubo et al., 1993). On the other hand, unlike fully functional auxiliary K^+ channel subunits, β- and γ-ENaCs do not form channels by themselves, but potentiate both I_{amil} and surface expression of these channels (Canessa et al., 1994; Firsov et al., 1996; Grunder et al., 1997; Awayda et al., 1997). In addition, both β- and γ-subunits are thought to contribute to the amiloride sensitivity of a heterotrimeric channel (Schild et al., 1997), although the sequence similar to the putative amiloride-binding domain identified in α-rENaC is found only in the γ-subunit (230WYKLHY235; positions indicated correspond to rat sequence). If the heterooligomeric assembly of the channel occurs because of the substitution of two α-subunits in a homotetramer for one β-subunit (which does not have the sequence) and one γ-subunit (which has this putative domain), the resulting $\alpha\beta\gamma$-ENaC should display a diminished amiloride sensitivity, similar to that observed in the experiments reported here, with the hybrid channels presumably composed of three WT subunits and one 278WYRFHY283-deletion mutant. The experiments in planar lipid bilayers and cell expression systems demonstrate that this not the case: the affinity of heterooligomeric ENaC for amiloride is within the same order of magnitude as that of a homooligomer (Canessa et al., 1993, 1994; Ismailov et al., 1996). On the other hand, McNicholas and Canessa (1997) demonstrated recently that the affinity of $\alpha\beta$ channels for amiloride was an order of magnitude lower than that of $\alpha\gamma$-ENaC and α-ENaC (Canessa et al., 1993; Ismailov et al., 1996) or $\alpha\beta\gamma$-ENaC (Canessa et al., 1994; Ismailov et al., 1996). These observations suggest that, at least in the heterotrimeric $\alpha\beta\gamma$-ENaC, the extracellular domains of α-, but not of β- and γ-subunits, are dominant in the formation of a putative amiloride-binding site outside the pore. It is thus possible that β- and/or γ-subunits do not substitute two α-subunits in the heterooligomer, but add on to the core conduction element formed by an α-tetramer. In other words, this argument may imply that tetrameric organization of ENaCs is only a minimal model for these channels.

The importance of such a proviso has been demonstrated by the recent report of Snyder et al. (1998), who conclude that the three ENaC subunits expressed in *Xenopus* oocytes assemble in a $3\alpha{:}3\beta{:}3\gamma$ ratio. However, taking into consideration that the methanethiosulfonate reagents ((2-aminoethyl)methanesulfonate hydrobromide, MTSEA, and [2-(trimethylammonium)ethyl]methanethiosulfonate bromide, MTSET) used in these combinatorial experiments of Snyder et al. are only partial inhibitors of WT and mutant ENaCs, respectively, the same data for $\alpha\beta\gamma$-ENaC can be fit to a hexamer formed by ~2 α-subunits, ~2 β-subunits, and ~2 γ-subunits, as follows. If one channel (either mutant, or WT) was completely insensitive to inhibition by a drug, and another was only partially sensitive (and the mutant was dominant), then, in a combinatorial approach, the fraction of macroscopic current that was attributed to fully WT (or fully mutant) channels in a WT:mutant mixture should be normalized for such finite sensitivity. In the absence of this correction, the fractions of channels at each WT:mutant DNA ratio were underestimated. For example, in the oocytes expressing only WT channels (i.e., when the WT: mutant ratio was equal to 1), the fraction of WT channels should be equal to unity (i.e., all observed channels are "insensitive" to MTSEA), but not to ~0.5 (which is the extent of inhibition). This, in turn, results in an overestimation of the number of subunits. Therefore, the number of individual subunits forming the channel should be less than the reported value of three (for each subunit), but more than one (because MTSEA-sensitive and MTSEA-insensitive fractions of current are distributed unequally in a 1:1 WT: mutant mixture).

In addition, the cell surface labeling experiments of Firsov et al. (1998) accentuate some potentially problematic uncertainties of cooperative cell surface expression of ENaC subunits. First, although the magnitude of an amiloride-sensitive current (I_{amil}) in oocytes correlated linearly with the specific binding of [^{125}I]anti-FLAG antibody to any two-epitope tag-labeled ENaC subunits ($\alpha^F + \beta^F$, $\alpha^F + \gamma^F$, or $\beta^F + \gamma^F$) of three expressed, the same data suggest that increasing the amount of RNA for the third (nontagged) subunit (while keeping the amount of RNA for two tagged subunits constant) decreases both the surface expression of channels and the I_{amil}. In the case of α- and β-subunits, this decrease was linear, whereas a 3- to 10-fold excess of γ-subunit cRNA over β and α ($1\alpha{:}1\beta{:}3\text{–}10\gamma$) produces a maximum of both I_{amil} and channel expression. Furthermore, specific binding of a monoclonal antibody to α- and γ-subunits calculated as an inverse slope of I_{amil}/binding plot in $\alpha^F\beta\gamma^F$ was an order of magnitude lower than that in $\alpha^F\beta^F\gamma$ and $\alpha\beta^F\gamma^F$. All of these findings are in contrast with the relative abundance of α-subunit over β- and γ-rENaC ($\alpha > \beta > \gamma$) expressed at the surface in these experiments when only one of three subunits was FLAG-epitope tagged.

There is at least one possible explanation that can help to reconcile tetrameric and higher order models, and it has been mentioned above in relation to the results of the combinatorial experiments reported here. Limited to the statistical analyses of functionally distinct phenotypes, this approach relies on the assumption that individual subunits exist as monomers. Therefore, the relative ratio of the operational units producing a functional change (term "fraction of WT or mutant protein" in Eq. 1) is considered an absolute number of elements in a multimer. This circumstance may lead one to underestimate the channel stoichiometry. For example, if both WT and mutant form dimers

by themselves, and each dimer is the minimum operational unit that produces a change of channel function (i.e., the substitution of WT for mutant occurs at the level of dimers), the results of functional combinatorial experiments will be off by a factor of 2 (or 3 for a trimeric unit, etc.). This may vary for different mutants. Given this possibility, the highest number determined for each subunit for different mutants means the best resolution, and this stoichiometry should be considered minimal.

In summary, we conclude that a core conduction element in cloned epithelial Na^+ channels (ENaCs) comprises at least four α-subunits. This conclusion is based on the results of two separate combinatorial studies performed in a cell-free planar lipid bilayer system, using two different mutant channel proteins, one with mutations affecting channel gating and another with mutations affecting the sensitivity of the channel to its specific inhibitor, amiloride.

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