Triple-barrel Organization of ENaC, a Cloned Epithelial Na\textsuperscript{+} Channel*

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A cloned rat epithelial Na\textsuperscript{+} channel (rENaC) was studied in planar lipid bilayers. Two forms of the channel were examined: channels produced by the $\alpha$ subunit alone and those formed by $\alpha$, $\beta$, and $\gamma$ subunits. The protein was derived from two sources: either from in vitro translation reaction followed by Sephadex column purification or from heterologous expression in Xenopus oocytes and isolation of plasma membranes. We found that either $\alpha$-rENaC alone or $\alpha$- in combination with $\beta$- and $\gamma$-rENaC, produced highly Na\textsuperscript{+}-selective ($P_{Na}/P_K = 10$), amiloride-sensitive ($K_{\text{amil}} = 170$ nM), and mechanosensitive cation channels in planar bilayers. $\alpha$-rENaC displayed a complicated gating mechanism: there was a nearly constitutively open 13-picosiemens (pS) state and a second 40-pS level that was achieved from the 13-pS level by a 26-pS transition. $\alpha$, $\beta$, $\gamma$-rENaC showed primarily the 13-pS level. $\alpha$-rENaC and $\alpha$, $\beta$-, $\gamma$-rENaC channels studied by patch clamp displayed the same gating pattern, albeit with $>2$-fold lowered conductance levels, i.e. 6 and 18 pS, respectively. Upon treatment of either channel in the sulfhydryl reducing agent dithiothreitol, both channels fluctuated among three independent 13-pS sublevels. Bathing each channel with a high salt solution (1.5 M NaCl) produced stochastic openings and closings of $19$ and $38$ pS in magnitude between all three conductance levels. Different combinations of $\alpha$, $\beta$, and $\gamma$-rENaC in the reconstitution mixture did not produce channels of intermediate conductance levels. These findings suggest that functional ENaC is composed of three identical conducting elements and that their gating is concerted.

An epithelial amiloride-sensitive Na\textsuperscript{+} channel has recently been cloned from rat distal colon (1–3) and, subsequently, from other tissues (4–7). This channel, termed rENaC for rat epithelial Na\textsuperscript{+} channel, consists of three homologous subunits $\alpha$, $\beta$, and $\gamma$. While the precise function of any of these subunits has not yet been determined, it appears that the conductive portion of the channel resides in the $\alpha$ subunit and that $\beta$ and $\gamma$ are necessary for enhanced ion channel activity in the Xenopus oocyte heterologous expression systems (2, 6, 7). It is also apparent that the channel’s gating properties are influenced by $\beta$ and $\gamma$, because truncations in the C-terminal region of these subunits produce a constitutive activation of the channel by increasing single channel open probability ($P_O$; Refs. 8 and 9).

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§ The abbreviations used are: pS, picosiemens; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; DTNB, 5,5-dithiobis(2-nitrobenzoate).
occurring after the 13-pS level was open. Moreover, upon treatment with the reducing agent dithiothreitol (DTT), both α-ENaC and α,β,γ-ENaC fluctuated among three independent 13-pS sublevels. We conclude that these channels are composed of three identical conduction elements and that the differences in the activity of the channels are modulated by the presence of the β and γ subunits within the complex.

MATERIALS AND METHODS

In Vitro Translation and Reconstitution of ENaC Subunits—The ENaC plasmids (pSPORT) were linearized overnight with NotI. The linearized DNA was purified using GeneClean kit (Bio101, La Jolla, CA), followed by in vitro transcription using T7 RNA polymerase according to the manufacturer's instructions (Ribomax kit, Promega Corp., Madison, WI). A 2:1 molar ratio of cap analog m7 G to the manufacturer's instructions (Ribomax kit, Promega Corp., Madison, WI). A 2:1 molar ratio of cap analog m7 G (NEB, Beverly, MA) to GTP was used in the translation reaction as described previously (20).

RNA was in vitro translated using a rabbit nucleic-acid-treated cell-free lysate system (Promega) according to the manufacturer's instructions and as described previously (20). 1.5 units of canine microsomal membranes (Promega) were added to the translation reaction.

In vitro translated proteins were purified on a G-75 Sephadex (Pharmacia Biotech Inc.) column as described previously (20). Fractions enriched in the applied ENaC subunits were reconstituted into phospholipid liposomes as described earlier (20). For liposomes containing α,β,γ-ENaC, subunits were in vitro translated separately, and each was purified over a gel filtration column. Identical elution fractions were collected and assayed for total 35S incorporation. The relative ratios of α, β, and γ subunits reconstituted into vesicles were determined by these 35S measurement.

Solutions containing similar membrane compositions for each were Control liposomes were also prepared from a mock in vitro translation/ Sephadex column purification run, following an identical protocol. In this case, ENaC RNA was simply omitted from the reaction mixture. These liposomes were used as control material for the bilayer experiments.

Channel Expression in Xenopus Oocytes and Oocyte Membrane Vesicle Preparation—Membrane vesicles from α-ENaC- and α,β,γ-ENaC mRNA-injected and water-injected oocytes were made as described by Perez et al. (21). Thirty oocytes in each group were rinsed and homogenized in high (K+)-sucreose medium containing multiple protease inhibitors. Membranes were isolated by discontinuous sucrose gradient centrifugation and resuspended in 300 mM sucrose, 100 mM/KCl, and 5 mM MOPS at pH 6.8. This material was aliquoted into 50-μl fractions and stored at −80 °C until use.

Bilayer Experiments and Data Analysis—Lipid bilayers were cast from a phospholipid solution in n-octane containing a 2:1:2 mixture of diphytanoyl-phosphatidylethanolamine/diphytanoyl-phosphatidylserine/ oxidized cholesterol (25 mg/ml). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Bilayer capacitances ranged from 300 to 400 picofarads. The solution bathing the bilayers consisted of 100 mM NaCl and 10 mM MOPS-Tris buffer (pH 7.4) unless otherwise noted. All solutions were made with Milli-Q water and were filter-sterilized through 0.22-μm Sterivex-GS filters (Millipore, Bedford, MA). The reconstituted vesicles or oocyte membranes were applied with a glass rod to one side (trans) of the preformed bilayer with the membrane voltage held at −40 mV. Under these conditions, channels oriented (90%) of the time, n = 250) asymmetrically, with the aspartate-sensitive (extracellular) side facing the trans solution. Voltage was applied to the cis chamber, and the trans chamber was virtual ground.

Single channel recordings were acquired and analyzed using pCLAMP software (Axon Instruments, Foster City, CA). The data were analyzed by the threshold-crossing technique employed to produce events lists. Open and closed dwell time histograms were logarithmically binned and fitted by a sum of exponential functions using maximum likelihood.

RESULTS

In vitro Translation of ENaC Channels in Bilayers—We have previously demonstrated that in vitro translated α-ENaC, the bovine ENaC homolog, migrates as a dimer on SDS-polyacrylamide gel electrophoresis under nonreducing conditions (7). As seen from the autoradiograph shown in Fig. 1 (lane 1), a similar gel pattern was observed for α-ENaC in vitro translated in the presence of microsomes and run under denatured but nonreduced conditions. In seven experiments of this type, the presence of a dimer present. If more than one channel was detected, the membrane was broken and the incorporation procedure repeated.

Patch Clamp Experiments—Cell-attached patch clamp recordings were obtained from defolliculated oocytes previously injected with α-ENaC and α,β,γ-ENaC rRNA (125 ng for oocytes expressing α-ENaC alone, and 1 ng of each subunit for oocytes expressing all three rENaC subunits) or 50 nl of nuclease-free water at room temperature. The composition of the pipette solution (ND-96) was (in mM): 96 NaCl, 2.4 KCl, 1.8 CaCl2, 1.8 MgCl2, and 5.0 HEPES (pH 7.4). The bathing solution had K+ substituted for Na+ to eliminate the resting membrane potential. Blunt-tipped patch microtipettes with a tip resistance of approximately 1–2 MΩ were fabricated using a Narashigi pp73 twostage micropipette puller. Under these conditions, inward single channel currents (downward current openings) in the cell-attached patches were carried by Na+ when the pipette was positive relative to the bath. Single channel currents were examined using an EPC-7 patch clamp amplifier (List Electronics, Darmstadt, Germany). After sealing the pipettes to the ool (seal resistance >10 MΩ), the cell-attached patches were clamped to a variety of voltages using an S-95 trilavole stimulus (Medical Systems; Sunnyvale, CA). All patch clamp experiments were performed at 21 ± 2 °C.
at 172 ± 11 kDa was always observed. However, the relative distribution of α-rENaC in the monomeric versus dimeric form differed from one batch of translation reaction to the other. The highest yield of a dimer to a monomer form is shown in the example in Fig. 1. Despite this variability there were never any observed differences in the biophysical properties of the in vitro translated channel incorporated into planar lipid bilayers (see below). Moreover, there was no evidence for the presence of a trimer form of α-rENaC (i.e. a product that migrated at ~270 kDa) was not observed; see lane 1). These observations suggest that the α subunit of ENaC may occur in its native state as a covalently linked dimer. As seen from lane 2, this pattern of interaction was not modified by the addition of the β and γ subunits. Lane 3 shows that the 180-kDa band is absent following reduction with 20 mM DTT and that the highest molecular mass band observed was one at 92 kDa. This size protein was expected for monomeric and core-glycosylated α-rENaC and was similar to previous observations on α-bENaC (7). Co-translation of all three subunits simultaneously did not reveal any forms between 200 and 300 kDa. Lane 4 shows a control, run in the absence of any ENaC message. Thus, the polypeptides seen in lanes 1–3 at molecular masses lower than 92 kDa probably represent partial translation products. The absence of a protein that migrated at a relative molecular mass in the range of the cumulative masses of one α, one β, and one γ subunit suggests that α, β, and γ do not covalently interact in a simple 1:1:1 ratio and that electrostatic interactions may be important in stabilizing the native ENaC complex.

Conductance Properties of rENaC—The column-purified in vitro translated polypeptides of α-rENaC, or α,β,γ-rENaC were reconstituted into liposomes and then incorporated into planar lipid bilayer membranes. As shown in Fig. 2, these in vitro translated proteins form functional ion channels (n = 7 for each). This figure depicts channel activity at different applied potentials. There was no effect of voltage on P0 of either channel. Fig. 2B shows the associated main state single channel current versus voltage curves for each channel. Both α-rENaC and α,β,γ-rENaC channels were linear. In over 1,000 attempted incorporations using control liposomes (see “Materials and Methods”), no channels of any kind were detected.

Examination of the single channel records over several minutes for both α-rENaC and α,β,γ-rENaC indicate continuous activity with no run-down (n = 16; Fig. 3). These channels were kinetically different from α-bENaC in bilayers, in that α-bENaC channel activity was punctuated by long closed periods lasting up to several minutes (20). Long (>1-s) closures were never observed for the rENaC channels in over 2 h of recording. α-rENaC channels displayed a specific gating pattern; with symmetrical 100 mM NaCl, the largest conductance level was 40 pS, but the channel appeared to fluctuate among 0-, 13-, 26-, and 40-pS levels (see associated all points amplitude histograms, Fig. 3B). These histograms did not fit the binomial algorithm for independent gating, indicating that these conductance levels cannot be produced by three independent channels. Neither the 13- nor 26-pS transitions were ever observed independently of each other in over 300 separate experiments. In the small number of cases (19/330) in which multiple channels were incorporated into the bilayer, the pattern shown at the top of Fig. 3 was simply repeated, i.e. for two channels in the bilayer, 4, 5, and 6 additional states were seen. Moreover, the single channel records indicated that the gating properties of the α-rENaC channel were not independent. Openings of the 26-pS level were only observed after the 13-pS state was open, never before. However, albeit infrequently, the 13-pS state would close prior to closure of the 26-pS transition (see Fig. 2A, top).

When α,β,γ-rENaC, in a 1:1:1 (w/w/w) combination, was incorporated into bilayers, only a 13-pS conductance level was observed (Figs. 2 and 3, bottom). However, brief openings of <250 ms in a duration to 40 pS were occasionally seen. Again, these openings occurred on the top of 13-pS conductance level that was, in essence, constitutively open. Event dwell time histograms were constructed for all the conductance levels of α-rENaC and α,β,γ-rENaC channels by setting a threshold at 6 pS for two channels in the bilayer, 4, 5, and 6 additional states were seen. Moreover, the single channel records indicated that the gating properties of the α-rENaC channel were not independent. Openings of the 26-pS level were only observed after the 13-pS state was open, never before. However, albeit infrequently, the 13-pS state would close prior to closure of the 26-pS transition (see Fig. 2A, top).

When α,β,γ-rENaC, in a 1:1:1 (w/w/w) combination, was incorporated into bilayers, only a 13-pS conductance level was observed (Figs. 2 and 3, bottom). However, brief openings of <250 ms in a duration to 40 pS were occasionally seen. Again, these openings occurred on the top of 13-pS conductance level that was, in essence, constitutively open. Event dwell time histograms were constructed for all the conductance levels of α-rENaC and α,β,γ-rENaC channels by setting a threshold at 50% of the open level of each substate (Fig. 3C). The dwell histograms in each sublevel for each channel were all fitted by a single exponential function. The closed state time constants were 70 ± 9 and 40 ± 5 ms for α-rENaC and α,β,γ-rENaC, respectively. The open state time constants for α-rENaC and α,β,γ-rENaC, respectively, were (in ms) 77 ± 10 and 72 ± 3 (13-pS state), 35 ± 8 and 51 ± 3 (26-pS state), and 91 ± 11 and 52 ± 3 (40-pS state). From this analysis, the time constant for
exit from the closed state is nearly twice as long for α-ENaC than for α,β,γ-ENaC.

Because α,β,γ-ENaC expressed in Xenopus oocytes displays a Na⁺-selective, 5-pS single channel conductance with relatively long open and closed conductance states as measured by patch clamp (2, 8), we wanted to assess directly whether bilayer reconstitution protocol utilizing in vitro translated polypeptides could affect conductance and/or open and closed times. Therefore, we compared ENaC single channel properties determined from patch clamp measurements of ENaC-expressing oocytes with those made in bilayers using in vitro translated proteins or subsequent to fusion of ENaC-expressing oocyte plasma membranes. Oocytes were injected either with α-ENaC or α,β,γ-ENaC cRNA and then either they were patch-clamped or their plasma membranes were used for fusion to planar bilayers. Fig. 4 shows the results of
these maneuvers. Patch clamp recordings of oocytes expressing αr-ENaC revealed channels with a large conductance of approximately 18 pS. Interposed among the large transitions were two additional conductance levels of 6 and 12 pS each. In approximately 18 pS. Interposed among the large transitions incorporated into bilayers. We conclude, therefore, that the measured by patch clamp, but only with larger conductance branes revealed channels with similar kinetic behavior to those made from oocytes expressing αENaC. We next examined whether these channels were comprised of more than one conductive type behavior and that α and γ themselves do not form channels, it is likely that the conduction pathway(s) of rENaC is formed by the α subunit(s). We next examined whether these channels were comprised of more than one conduction element. The results presented in Fig. 1 indicate that disulfide bond formation occurs between two of the subunits. Thus, we postulated that disrupting disulfide bonds would reveal more fundamental kinetic behavior. Therefore, we tested the effects of 25 μM of the reducing agent DTT on αr-ENaC and α,β,γ-rENaC in bilayers. Fig. 7 summarizes the results of a typical experiment. First, either ENaC was sensitive to DTT only from the trans (i.e., outside) bathing solution. Second, concentrations of DTT below 25 μM had no effect on single channel properties, and concentrations larger than 50 μM irreversibly damaged coordinated channel activity (data not shown). As can be seen in the figure, treatment of either αr-ENaC or α,β,γ-rENaC with DTT resulted in the appearance of three indistinguishable 13-pS conductance states. As β and γ, independently or together, do not induce channel activity, at least as expressed in oocytes (2, 7), it is plausible that both αr-ENaC and α,β,γ-rENaC consist of a minimum of three protocanels formed by α subunits that gate in a concerted fashion. In the presence of DTT, this synchronous gating may be disrupted, thus permitting independent operation of these three conductive elements.

To further test the hypothesis that ENaC consists of three individual protochannels formed by α subunits, we cross-linked all of the subunits present in the functional complex together.

Ion Selectivity of rENaC in Planar Bilayers—When α-rENaC or α,β,γ-rENaC channels were bathed with asymmetric solutions of NaCl (a 10-fold gradient) under nonstretched conditions, a reversal potential of 57 ± 3 mV was measured, and a permeability ratio for Na⁺ versus Cl⁻ of 10:1 for each channel type was calculated. These values did not change upon stretch (n = 9 for each channel type). These results are similar to those previously reported for a-bENaC (20). Likewise, P₉Na/P₉Cl for each channel was determined from reversal potential measurements made under biionic conditions in the absence of and in the presence of a 0.26 mm Hg hydrostatic pressure gradient across the membrane (Fig. 6). Under nonstretched conditions, both channels were 10-fold more permeable to Na⁺ than to K⁺. However, upon stretch, each channel lost some of its ability to discriminate between Na⁺ and K⁺, decreasing to 3:1 and 4:1 for α-rENaC and α,β,γ-rENaC, respectively.

Gating Properties of rENaC—The records depicted in Figs. 2 and 3 indicate that the apparent single channel kinetic behavior of α,β,γ-rENaC was different from that of αr-ENaC. αr-ENaC has one small 13-pS conductance level that is almost continuously open and a larger 40-pS level, while α,β,γ-rENaC primarily displays the 13 pS level with only brief openings to the 40-pS level. Based on the observations that both αr-ENaC and α,β,γ-rENaC display kinetic properties indicative of sub-conductive type behavior and that β and γ themselves do not form channels, it is likely that the conduction pathway(s) of rENaC is formed by the α subunit(s). We next examined whether these channels were comprised of more than one conduction element. The results presented in Fig. 1 indicate that disulfide bond formation occurs between two of the subunits. Thus, we postulated that disrupting disulfide bonds would reveal more fundamental kinetic behavior. Therefore, we tested the effects of 25 μM of the reducing agent DTT on αr-ENaC and α,β,γ-rENaC in bilayers. Fig. 7 summarizes the results of a typical experiment. First, either ENaC was sensitive to DTT only from the trans (i.e., outside) bathing solution. Second, concentrations of DTT below 25 μM had no effect on single channel properties, and concentrations larger than 50 μM irreversibly damaged coordinated channel activity (data not shown). As can be seen in the figure, treatment of either αr-ENaC or α,β,γ-rENaC with DTT resulted in the appearance of three indistinguishable 13-pS conductance states. As β and γ, independently or together, do not induce channel activity, at least as expressed in oocytes (2, 7), it is plausible that both αr-ENaC and α,β,γ-rENaC consist of a minimum of three protocanels formed by α subunits that gate in a concerted fashion. In the presence of DTT, this synchronous gating may be disrupted, thus permitting independent operation of these three conductive elements.

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The sulfhydryl reactive reagent 5,5-dithiobis(2-nitrobenzoate) (DTNB) was used as the cross-linking reagent. Again, DTNB was only effective from the trans side of the bilayer. Fig. 5 shows that DTNB treatment of either α-rENaC or αβγ-rENaC produced channels that fluctuated between a 0- and 40-pS level. Thus, the kinetic behavior of the channels indicated that the three putative individual α subunit proto-channels may operate in concert. However, the complete opening of this channel complex when in its native form occurred in two steps, the second twice the size of the first (Fig. 3A). Thus, we hypothesized that one of the α subunit protochannels was anchored to the complex by a noncovalent interaction. To test this idea, we exposed rENaC to elevated salt concentrations in the hope of minimizing electrostatic interactions between subunits (the bulk of the amino acids comprising each ENaC subunit lies in a large extracellular loop (24–26)). Thus, the prediction was that both α-rENaC and αβγ-rENaC should gate in a very similar manner, with one of the protochannels behaving as an independent lower conductance channel and the two disulfide-linked protochannels operating in effect as a single higher conductance unit. This experiment has been performed a total of six times each for α-rENaC and αβγ-rENaC, with identical results.

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To better understand the biophysical consequences of DTT and high salt treatment of rENaC, amplitude histogram analyses of current records made under these experimental conditions were performed. All points and events amplitude histograms are presented in Fig. 9. The overall distribution of the all
points amplitude histograms was binomial, thus suggesting equal probability of the channel residing in any conductance level (cf., Fig. 3B). The events amplitude histogram revealed a disruption of concerted gating following DTT or high salt treatment in that transitions to all conductance levels occurred independently. Such an outcome of events amplitude histogram may be due to counting channel openings “from” a fixed zero current level “to” a conductance sublevel. In order to overcome this limitation we have constructed amplitude histograms with a gradually sliding zero level, the level that channel resides in becomes an “apparent zero-current level” for the next transition. This maneuver permits the construction of a histogram of absolute values of amplitudes of transitions. The resulting histograms (Fig. 10) show that the predominant transition in the case of DTT-treated rENaC was 13 pS, while high salt treatment produced, in equal probability, transitions of 19 and 38 pS. Taking into account that the increased conductance of the channels in this latter case was due to elevated [Na⁺], these results support the hypothesis that rENaC consists of a minimum of three conductive elements, two of which may be linked by disulfide bonds and the third noncovalently anchored to the covalently linked complex. Histograms for DTT and high salt-treated α-rENaC and αβγ-rENaC were almost identical (Figs. 9 and 10), indicating that the conductive pore of these channels was formed by α-rENaC.

Effect of Amiloride on rENaC—The effect of amiloride on α-rENaC and αβγ-rENaC is summarized in the dose-response curves presented in Fig. 11. Amiloride inhibited both of these channels with very similar efficacy. The apparent amiloride-inhibitory constant (Kᵢamil) was 170 ± 25 nM (n = 12) for both channels. Treatment of α-rENaC or αβγ-rENaC with either DTT or DTNB had no significant effect on Kᵢamil. Likewise, bathing either α-rENaC or αβγ-rENaC with symmetrical 1.5 M NaCl solutions had only a minor effect on Kᵢamil, shifting it to 250 ± 30 nM (n = 3) and 230 ± 20 nM (n = 3) for each channel, respectively, consistent with amiloride acting as a competitive inhibitor of these channels (18, 27). The best fit of these amiloride dose-response curves was achieved with a Michaelis-Men-
properties, ten formalism using a Hill coefficient of 3. All other channel 
state behavior were preserved in the bilayer. Because amiloride-sensitive Na
ion selectivity, amiloride sensitivity, and sub-
unit is comprised of a minimum of three conductive elements
components may modify the resultant single channel kinetic
properties and/or conductance. Table I presents the results of
these experiments. First, \( \beta \) or \( \gamma \) alone or in combination, do not
produce channels of any kind, confirming the observations
initially made in oocytes (2, 6, 7). Likewise, \( \alpha \beta \) or \( \alpha \gamma \) yielded
channels identical to \( \alpha \)-rENaC described above. An excess of \( \beta \)
and \( \gamma \) relative to \( \alpha \) in the reconstitution mixture produced
channels indistinguishable from those of \( \alpha \beta \gamma \) in a 1:1:1 ratio.
If \( \alpha \) exceeded \( \beta \) and \( \gamma \) by more than 7-fold, only \( \alpha \)-type channels
were seen. Although these experiments do not address the actual
stochiometry of the subunit composition of functional
rENaC, they do, nonetheless, suggest that the variable conduc-
tances observed in different cells and tissues cannot be attrib-
uted solely to different ratios of ENaC subunits comprising the
functional channel complex.

**TABLE I**

| Combination | rENaC channel type | n  |
|-------------|--------------------|----|
| 1:0:0       | \( \alpha \)        | 25 |
| 0:1:0       | \( \alpha \)        | (0/250) |
| 0:0:1       | \( \alpha \)        | (0/250) |
| 0:1:1       | \( \alpha \)        | (0/250) |
| 1:1:1       | \( \alpha \beta \gamma \) | 9  |
| 1:1:0       | \( \alpha \)        | 4  |
| 1:0:1       | \( \alpha \)        | 3  |
| 1:1:1       | \( \alpha \beta \gamma \) | 3  |
| 1:1:1       | \( \alpha \beta \gamma \) | 3  |
| 3:1:1       | \( \alpha \beta \gamma \) | 3  |
| 3:1:1       | \( \alpha \beta \gamma \) | 3  |
| 7:1:1       | \( \alpha \beta \gamma \) | 5  |
| 15:1:1      | \( \alpha \)        | 3  |
| 22:1:1      | \( \alpha \)        | 4  |
| 30:1:1      | \( \alpha \)        | 4  |
| 40:1:1      | \( \alpha \)        | 3  |
| 60:1:1      | \( \alpha \)        | 3  |
| 100:1:1     | \( \alpha \)       | 3  |

**FIG. 10.** Events amplitude histograms of DTT and high salt-
treated \( \alpha \)-rENaC and \( \alpha \beta \gamma \)-rENaC, using a “sliding” zero-current level. Experimental conditions were the same as described in the
legends to Figs. 4 and 5, respectively. Events lists were produced by
pCLAMP software using 50% amplitude threshold (with 3-ms duration)
technique. Transitions from 0-pS level, from 13-pS level, from 26-pS
level, and from 40-pS level were sorted manually in a pStat events list
spreadsheet session and processed by the pStat routine to produce
events amplitude histograms.

**FIG. 11.** Amiloride dose-response curves of in vitro translated
\( \alpha \)-rENaC and \( \alpha \beta \gamma \)-rENaC in planar lipid bilayers under different
experimental conditions. Points in plots are mean ± S.D. for at
least six experiments under each condition. Amiloride was sequentially
added in increasing concentrations to the
bathing solution. Our observations, made both in
patch clamp studies of rENaC-expressing oocytes and in planar
bilayers, indicate that \( \alpha \)-rENaC and \( \alpha \beta \gamma \)-rENaC display characteristic single channel properties; namely, \( \alpha \)-rENaC consisted of a small (13 pS in bilayers, 6 pS in patch) and a large
(2 times the conductance of the small) conductance state, while
\( \alpha \beta \gamma \)-rENaC primarily exhibited only the small conductance
level. Because amiloride-sensitive Na⁺ channels recorded in
both native epithelial and cultured cells exhibit a wide range of
single channel conductances (17, 18), and because different
relative levels of \( \alpha \), \( \beta \), \( \gamma \)-rENaC mRNA have been detected
within a given tissue (15, 16, 28), we utilized the in vitro
translation-bilayer reconstitution system for rENaC subunits to test the hypothesis that varying ratios of rENaC subunit
components may modify the resultant single channel kinetic
properties and/or conductance. Table I presents the results of
these experiments. First, \( \beta \) or \( \gamma \) alone or in combination, do not
produce channels of any kind, confirming the observations
initially made in oocytes (2, 6, 7). Likewise, \( \alpha \beta \) or \( \alpha \gamma \) yielded
channels identical to \( \alpha \)-rENaC described above. An excess of \( \beta \)
and \( \gamma \) relative to \( \alpha \) in the reconstitution mixture produced
channels indistinguishable from those of \( \alpha \beta \gamma \) in a 1:1:1 ratio.
If \( \alpha \) exceeded \( \beta \) and \( \gamma \) by more than 7-fold, only \( \alpha \)-type channels
were seen. Although these experiments do not address the actual
stochiometry of the subunit composition of functional
rENaC, they do, nonetheless, suggest that the variable conduc-
tances observed in different cells and tissues cannot be attrib-
uted solely to different ratios of ENaC subunits comprising the
functional channel complex.

**DISCUSSION**

In this work, we report the successful incorporation of
\( \alpha \)-rENaC and \( \alpha \beta \beta \gamma \)-rENaC into planar lipid bilayers. ENaC
protein was obtained either from a rabbit reticulocyte lysate in
vitro translation system or following expression in Xenopus
oocytes and isolation of oocyte plasma membranes. The results
obtained using either of these preparations were identical. Our
experiments also indicate that the \( \alpha \)-rENaC subunit alone or in combination with other \( \alpha \) subunits acts as the conductive
element of the channel complex. However, a high degree of
concerted gating occurs between these putative conduction elements and those covalently linked by disulfide bonds. The kinetic behavior of ENaC suggests that a functional channel
unit is comprised of a minimum of three conductive elements
formed by \( \alpha \) subunits. ENaCs are highly Na⁺-selective, are
inhibited with high affinity by the diuretic amiloride, and are
mechanosensitive.

Comparison of rENaC in Bilayers and by Patch Clamp—A
number of biophysical experiments were performed on
\( \alpha \)-rENaC and \( \alpha \beta \gamma \)-rENaC to compare their properties when
expressed in Xenopus oocytes and when purified and reconsti-
tuted into planar lipid bilayers. For \( \alpha \)-rENaC, the overall ki-
etic behavior of the channel was similar in patch clamp and
bilayer experiments, namely a small conductance level on top
of which another conductance level (twice the size of the small
one) would open. The absolute value of the small conductance
level differed (13 pS in the bilayer and 6 pS in the patch). For α-ENaC and αβγ-ENaC, a 6-pS (patch) or 13-pS (bilayers) level was routinely measured. That these amiloride-sensitive channels expressed in oocytes are ENaCs is supported by the fact that they were never observed in water-injected oocytes. We conclude, therefore, that the microenvironment in which ENaC resides determines in large measure its conductance and mean open and closed times (31–33). Aside from these changes, the channels displayed comparable amiloride sensitivities, ion selectivities, and gating patterns.

The existence of subconductive levels within a single ion channel has been reported for many ion channels including the acetylcholine receptor (34), the glycine, GABA, and glutamate receptors (35–37), the dihydropyridine-sensitive Ca^{2+} channel (38), inwardly rectifying K^{+} channels (39, 40), the ryanodine receptor cation channel (41), and gramicidin (33). It is not clear why subconductive behavior has not been observed in patch records of αβγ-ENaC channels. One possible explanation is that these channels have not yet been analyzed at high time resolution. Another reason may be that upon drawing the oocyte membrane into the tip of a patch electrode, sufficient tension may have already been applied to produce what appear to be three independent, small conductance channels (cf., Figs. 3 and 4; Refs. 2 and 8). The fine details of channel conductances appear to be influenced by the methods of observation.

**Kinetic Behavior of ENaC in Bilayers—** Visual inspection of α-ENaC transitions in bilayers reveals that the channel fluctuates either between 0 and 13 pS or between 13 and 40 pS but never (in at least 2 h of recording) between 0 and 26 pS. Residence of the channel in its 26-pS level was rare and short lived and occurred only when the channel transited from its 40-pS level. Residence of the channel in its 26-pS level was rare and short lived and occurred only when the channel transited from its 40-pS level. Residence of the channel in its 26-pS level was rare and short lived. Under these conditions, the channel flickered between 0 and 13 pS, and 0 and 26 pS and episodically reached its 40-pS level. Likewise, when α-ENaC was treated with DTT, transitions occurred independently between all three equally spaced conductance levels. High salt or DTT treatment of αβγ-ENaC produced an identical pattern of channel activity as for α-ENaC (Figs. 5 and 6).

Both high salt and DTT disrupt protein–protein interactions. Thus, the change in biophysical properties associated with these treatments implies that a multimeric form of α-ENaC underlies channel behavior. Because these three levels represent subconductive states of a single channel entity (23), this kinetic behavior strongly suggests that ENaC is composed of a minimum of three conductive elements and that a pore is formed within each one of these elements. The observations that the same kind of channel activity following DTT treatment is seen for ENaC composed of only α or of αβγ and that β and γ cannot form ion channels by themselves (Table 1) suggest that the conduction element is the α subunit of ENaC. Whether a monomer or dimer (or higher form) of 2α-ENaC acts as the unit conduction element cannot be deduced from these experiments.

As a first approximation, a simple kinetic model of ENaC can be described as follows:

\[
\begin{align*}
C & \overset{k}{\underset{l}{\rightleftharpoons}} \frac{O_1}{k_1} \overset{m}{\underset{l_1}{\rightleftharpoons}} \frac{O_2}{(13\ pS)} \overset{m_1}{\underset{l_1}{\rightleftharpoons}} \frac{O_3}{(40\ pS)}
\end{align*}
\]  
\[\text{(Eq. 1)}\]

or

\[
\begin{align*}
C & \overset{k}{\underset{l}{\rightleftharpoons}} O_1 \overset{m}{\underset{l_1}{\rightleftharpoons}} O_2 \overset{m_1}{\underset{l_1}{\rightleftharpoons}} O_3
\end{align*}
\]

\[\text{(0 pS)} \overset{k_1}{\underset{l_1}{\rightleftharpoons}} \frac{13\ pS}{(13\ pS)} \overset{m_1}{\underset{l_1}{\rightleftharpoons}} \frac{26\ pS}{(26\ pS)} \overset{m_1}{\underset{l_1}{\rightleftharpoons}} \frac{40\ pS}{(40\ pS)}
\]

\[\text{(Eq. 2)}\]

where C represents the closed state and O_1, O_2, and O_3 the 13-, 26-, and 40-pS open states, respectively. As indicated above, there were only a few transitions to 26 pS that were observed, and these only occurred from the 40-pS conductance level and had a time constant of 35 ± 8 ms (Fig. 2, B and C). Three possible explanations can account for these data: 1) if the 26-pS transition is comprised of two concertedly linked 13-pS openings, it may be that there is simply a short-lived half-closed state associated with the closing and opening of this 26-pS level; 2) if opening of the 13-pS level is required for the subsequent opening of the 26-pS level, it may be that a brief transient closing of the 13-pS level triggers the closing of the 26-pS level; and 3) if a 13-pS level transiently dissociates from the complex in the lipid bilayer, a 26-pS level may be observed. The first possibility would predict zero residence in the 26-pS level, assuming that closure of the double protochannel was reversible. This second explanation does not account for the transition from 13 through 26 to 40 pS. If the third possibility were true, a transition from 0 to 26 pS would be expected, but the data do not support this. Thus, we simplified the scheme to contain two predominant transitions: one of 13 pS and the other 26 pS in size. When both are open, the conductance of the channel is 40 pS.

There is certain probability p that the channel will reside in any of the given states, and because at any given time the channel must be in one of them, the sum of these probabilities must equal one.

\[p_C + p_{O_1} + p_{O_2} = 1\]

\[\text{(Eq. 3)}\]

Also, for a system in equilibrium the percentage of channels in any given state must remain constant. Therefore, the rate of transition out of one state must equal the rate of transition into it. The constants k, m_1, and m_2 are measures of transition rates between these states. Therefore, the net transition rate out of a state is the product of the rate constant and the probability of the channel being in that state.

\[k_P_C = k_1 P_{O_1}\]

\[\text{(4)}\]

\[k_P_{O_1} = k_1 P_C + m P_{O_1}\]

\[\text{(5)}\]

\[m P_{O_2} = m P_{O_1}\]

\[\text{(6)}\]

If the values for each of the rate constants are determined, it will be possible to calculate the values for the probabilities P_C, P_{O_1}, and P_{O_2} using Equations 3, 4, and 5. The probability of the channel residing in a given state for time t or less is as follows,

\[F(t) = 1 - e^{-kt}\]

\[\text{(7)}\]

where T equals one divided by the sum of all of the rate constants leading away from the state (42). Thus,

\[F_C(t) = 1 - e^{-kt}\]

\[F_{O_1}(t) = 1 - e^{-k_1 t}\]

\[F_{O_2}(t) = 1 - e^{-m_1 t}\]

\[\text{(8)}\]

\[\text{(9)}\]

\[\text{(10)}\]

Substituting with the experimental data (Fig. 2C), we can calculate the following: k = 145 s^{-1}; k_1 + m = 13.5 s^{-1}; and m_1 = 11.5 s^{-1}.

This gives a unique solution for the rate constants k and m_1. However, to complete the model, we need values for k_2 and m_2. The appropriate equation can be obtained by calculating the probability of the channel proceeding to the 40-pS state from
the 13-PS state (i.e. \( P_{O_1} \rightarrow O_1 \)). This has been calculated from 10 min of single channel recording by counting the number of times that the channel switched from the 13- to the 40-PS state and dividing by the total number of times the channel switched out of the 13-PS state. This probability was found to be \( 0.66 \pm 0.05 \). We know that this probability must equal the rate of transition into the O3 state divided by the total rate of transition out of the O2 state.

\[
P_{O_1 \rightarrow O_2} = \frac{m(k_1 + m)}{k_1} \quad \text{(Eq. 1)}
\]

Thus, we calculate the values of the rate constants \( k_1 = 4.95 \) s\(^{-1}\) and \( m = 9.0 \) s\(^{-1}\). Using these calculated values, we can now solve equations 3, 4, and 6 to calculate the probability of the channel being in any of the possible three states: \( P_{O_1} = 0.14; P_{O_2} = 0.40; \) and \( P_{O_3} = 0.46. \)

From the all points amplitude histograms (Fig. 3B), we can compute the probability of finding a channel in any given state by calculating the area under each individual curve and dividing by the total area under the histogram. This analysis yields the following results: \( P_{O_1} = 0.14 \pm 0.05; P_{O_2} = 0.35 \pm 0.05; \) and \( P_{O_3} = 0.51 \pm 0.05. \)

A comparison of the values for the probabilities calculated from the histogram analysis and those derived from the kinetic simulation are in good agreement. This simulation thus formalizes the kinetic behavior of a triple-barrel model for ENaC. This triple-barrel model of ENaC is similar to that proposed for inwardly rectifying K\(^+\) channels (39, 40). Interestingly, while there is little homology between ENaC and IRK1 or ROMK1 at the nucleotide and amino acid levels (43, 44), the membrane topology of both classes of ion channel are similar in that they each have only two putative membrane-spanning domains (24–26).

Are BioPhysically Distinct Amiloride-sensitive Na\(^{+}\) channels Referrable to Different Combinations of \( \alpha, \beta, \) and \( \gamma \) Subunits?—Steroid hormones increase transepithelial Na\(^{+}\) transport in target epithelia such as colon, lung, and renal cortical collecting tubule (45). These tissues contain message for all three subunits of ENaC (2, 5, 6, 15, 16, 46). Steroid hormones have also been found to increase the abundance of \( \alpha\)-ENaC mRNA relative to \( \beta \) and \( \gamma \) in lung (28, 30). In rats fed a low Na\(^{+}\) diet to elevate circulating aldosterone levels, rENaC does not produce amiloride-sensitive Na\(^{+}\) channels have revealed a different pattern of polypeptide composition of the channel complex, depending upon the source material (47–49). However, a thorough analysis of variations in \( \alpha, \beta, \) and \( \gamma \) subunit ratios on single channel properties will only be achieved once the functional significance of any channel-associated proteins are elucidated.

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