# Gating of Amiloride-Sensitive $Na^+$ Channels: Subunit-Subunit Interactions and Inhibition by the Cystic Fibrosis Transmembrane Conductance Regulator

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ABSTRACT In search of the structural basis for gating of amiloride-sensitive $Na^+$ channels, kinetic properties of single homo and heterooligomeric ENaCs formed by the subunits with individual truncated cytoplasmic domains were studied in a cell-free planar lipid bilayer reconstitution system. Our results identify the N-terminus of the $\alpha$-subunit as a major determinant of kinetic behavior of both homooligomeric and heterooligomeric ENaCs, although the carboxy-terminal domains of $\beta$- and $\gamma$-ENaC subunits play important role(s) in modulation of the kinetics of heterooligomeric channels. We also found that the cystic fibrosis transmembrane conductance regulator (CFTR) inhibits amiloride-sensitive channels, at least in part, by modulating their gating. Comparison of these data suggests that the modulatory effects of the $\beta$- and $\gamma$-ENaC subunits, and of the CFTR, may involve the same, or closely related, mechanism(s); namely, "locking" the heterooligomeric channels in their closed state. These mechanisms, however, do not completely override the gating mechanism of the $\alpha$-channel.

## INTRODUCTION

Genetic linkage analyses of mutations found in the three polypeptides comprising amiloride-sensitive epithelial $Na^+$ channels ($\alpha$-, $\beta$-, and $\gamma$-ENaC)s, cloned originally from the colon of salt-depleted or treated with dexamethasone rats (Canessa et al., 1993, 1994; Lingueglia et al., 1993) provided a breakthrough in the elucidation of the molecular basis of important human genetic diseases, such as salt-sensitive hypertension (Liddle's syndrome; Shimkets et al., 1994; Hansson et al., 1995a, b), and salt-wasting syndrome (pseudohypoaldosteronism type 1; Chang et al., 1996). Combined with functional electrophysiological experiments in heterologous cell expression systems, these studies identified the amino-terminal domain of the $\alpha$-subunit and the carboxy-terminal domains of $\beta$- and $\gamma$-ENaCs as being responsible for the "loss-of-function" and the "gain-of-function" of amiloride-sensitive $Na^+$ channels, respectively (Schild et al., 1995; Firsov et al., 1996; Gründer et al., 1997). Both abnormalities of channel function were attributed, at least in part, to defects in channel gating which, in effect, result in altered channel open probability.

The present study was performed to systematically examine the roles of cytoplasmic domains of individual ENaC subunits in determining kinetic properties of these channels, taking advantage of the cell-free in vitro translation/planar lipid bilayer reconstitution approach (Rosenberg and East, 1992). This technique has proven its unique utility, particularly with respect to ENaCs, allowing detailed studies of biophysical properties of the putative core conduction element made by the $\alpha$-subunit alone (Berdiev et al., 1998), and those of the channels comprised of different combinations of all three subunits (Ismailov et al., 1999). By using this system we have been able to demonstrate that 1) consistent with the hypothesis of Gründer et al. (1997) proposed originally to explain the loss of function in pseudohypoaldosteronism type 1, kinetic properties of the homooligomeric channels formed by the amino-terminally truncated mutant $\alpha_{\Delta 2-109}$-rENaC were quite different from those of the channels formed by the wild-type (WT) $\alpha$-rENaC (Berdiev et al., 1998); 2) heterooligomeric channels formed by the WT $\alpha$-rENaC and the carboxy-terminally truncated $\beta$- and $\gamma$-subunits ($\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta C}$-rENaCs) display continuous activation (Ismailov et al., 1999), characteristic of the Liddle's disease phenotype (Firsov et al., 1996); and 3) synthetic peptides with the sequences comprising the distal 30-residue domains of carboxy-termini in the $\beta$- and $\gamma$-hENaC added to the solution bathing the presumptive cytoplasmic face of the $\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta C}$-rENaCs decreased open probability of these continuously active channels by changing the time spent by the channels in their closed state (Ismailov et al., 1999). In this report we present the results of a systematic study of kinetic properties of single $\alpha\beta\gamma$-rENaC formed by the subunits with individual cytoplasmic tails truncated (in combination with the WT subunits, and/or with each other). To better determine functional significance of different structural domains, we correlate our kinetic data with the effects of the cystic fibrosis transmembrane conductance regulator (CFTR), which has been shown to inhibit heterooligomeric ENaCs in several different cell expression systems (Stutts et al., 1995; 1997; Ling

*Received for publication 26 August 1999 and in final form 30 December 1999.*

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0006-3495/00/04/1881/14 $2.00

et al., 1997; Mall et al., 1996; Kunzelmann et al., 1997; Briel et al., 1998; Chabot et al., 1999), and in planar lipid bilayers (Ismailov, 1996b; 1997a).

Our results suggest that 1) the N-terminus of the $\alpha$-subunit is a major determinant of gating of both homooligomeric and heterooligomeric ENaCs; 2) the carboxy-terminal domains of $\beta$- and $\gamma$-ENaC subunits play an important role in modulation of the kinetics of heterooligomeric channels; but 3) they do not completely override the effects of the $\alpha$-N-terminus on the gating mechanism of the heterooligomeric channel; and 4) CFTR inhibits amiloride-sensitive channels, at least in part, by modulating their gating, using the same (or closely related) mechanism as that involved in the modulation of the kinetics of heterooligomeric channels by carboxy-terminal domains of $\beta$- and $\gamma$-ENaC subunits.

## MATERIALS AND METHODS

### Constructs and reagents

The N-terminally truncated $\beta_{\Delta N}$- and $\gamma_{\Delta N}$-rENaCs were constructed by the PCR method as follows. A 797-bp fragment was generated using $\beta$-rENaC cDNA as a template and a sense primer CGACGTCGACCATGGCCATGTGGTTCCTGCTCACGCTGC corresponding to nucleotides 148–173 of $\beta$-rENaC, and an anti-sense primer GTGGACGCAAGGAAGGGGACATAG corresponding to nucleotides 909–932 of $\beta$-rENaC. A 692-bp fragment was synthesized using $\gamma$-rENaC cDNA as a template with a sense primer CGACGTCGACCATGCTGCTGTGGATCGCGT corresponding to nucleotides 160–175 of $\gamma$-rENaC, and an anti-sense primer TTGGATGGTGGAAAAGCGTGAAGT corresponding to nucleotides 815–838 of $\gamma$rENaC. The sense primers also contained an upstream *Sal*I restriction site, a partial Kozak consensus site sequence, and an initiator methionine codon. The amplified PCR products for $\beta$- and $\gamma$-constructs contained a *Psh*AI restriction site, and an *Mfe*I (*Mun*I) restriction site, respectively, located just upstream of the respective anti-sense primer sequence. PCR amplification of either construct was performed as follows: denaturing the reaction mixture at 94°C (1 min) was followed by 2 cycles (1 min at 94°C, 1 min at 60°C, and 2 min at 72°C), 24 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C), and a final extension at 72°C for 10 min. Then, the 797-bp PCR product of $\beta_{\Delta N}$-rENaC and the 692-bp PCR product of the $\gamma_{\Delta N}$-rENaC were isolated, purified (Wizard, Promega, Madison, WI), and subcloned into pcR 2.1 (TA Cloning Kit, Invitrogen, Carlsbad, CA). The sequences were verified by ABI PRISM dye terminator cycle sequencing (Perkin-Elmer, Norwalk, CT); $\Delta$2–49-$\beta$rENaC was constructed by digesting pSport/$\beta$-rENaC and the subcloned PCR product with *Sal*I/*Psh*AI and ligating the gel-purified PCR fragment into the digested pSport/$\beta$-rENaC vector. The $\Delta$2–53-$\gamma$rENaC was constructed by digesting pSport/$\gamma$-rENaC and the subcloned PCR product with *Sal*I/*Mfe*I and ligating the gel-purified PCR fragment into the digested pSport/$\gamma$-rENaC vector. The sequences of both strands of each construct were confirmed by ABI PRISM dye terminator cycle sequencing. Thus, in $\Delta$2–49-$\beta$-rENaC the N-terminal 49 amino acids were deleted and Lys-49 replaced by a methionine, and in $\Delta$2–53-$\gamma$-rENaC the N-terminal 53 amino acids were deleted and P53 was replaced by a methionine. The N-terminally truncated $\alpha_{\Delta N}$-rENaC was made as described previously (Berdiev et al., 1998), and the C-terminally truncated $\beta_{\Delta C}$- and $\gamma_{\Delta C}$-rENaC were a kind gift of Dr. B. Rossier (Lausanne, Switzerland). CFTR immunopurified from bovine tracheal epithelia as described in Jovov et al. (1995) was kindly provided by Dr. D. J. Benos (UAB). A purified catalytic subunit of protein kinase A was a gift of Dr. G. Johnson (UAB). 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 $\mu$m filter, Millipore Corp., Bedford, MA).

### In vitro translation and reconstitution of rENaC proteins

Individual rENaC proteins were in vitro-translated by using a TNT transcription-translation kit (Promega, Madison, WI) according to manufacturer's instructions in the presence of canine microsomal membranes (Promega) and 0.8 mCi/ml [$^{35}$S]Trans label (ICN, Costa Mesa, CA). A 25-$\mu$l translation reaction for each cRNA 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 $\mu$l 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., Inc., Piscataway, NJ) gel filtration column with a buffer containing 500 mM NaCl, 1 mM EDTA, and 10 mM Tris (pH 7.6), and reconstituted into proteoliposomes at an equal molar ratio based on the [$^{35}$S] methionine/cysteine labeling for each subunit. Proteoliposomes were sonicated for 40–45 s at 43 kHz (160 W) in the presence of 50 $\mu$M DTT, and allowed to re-form by freeze-thawing three to five times. This protocol was developed to dissociate putative individual conduction elements of ENaC held together by sulfhydryl bonds, and results in recordings of single amiloride-sensitive $Na^+$ selective channels with uniform conductance of 13 pS in over 70% of total incorporations (Berdiev et al., 1998; Ismailov et al., 1999). Mock controls were prepared by performing the in vitro translation reaction in the absence of rENaC cRNA, and reconstituting the purified reaction products into proteoliposomes following an identical protocol.

### Planar lipid bilayer experiments

Proteoliposomes were fused with the Mueller-Rudin planar lipid bilayers made of a 2:1 (wt/wt) diphytanoyl-phosphatidyl-ethanolamine/diphytanoyl-phosphatidylserine solution in n-octane (final lipid concentration 25 mg/ml). The bilayers were bathed with symmetrical 100 mM NaMOPS, 10 mM Tris buffer (pH 7.4). Single channel currents were measured using a conventional current-to-voltage converter with a 1 G$\Omega$ feedback resistor (Eltec, Daytona Beach, FL) as described previously (Ismailov et al., 1996a, 1997b). Identity of ENaCs was tested in the end of each experiment by adding 0.5 $\mu$M amiloride to the *trans* compartment of the bilayer chamber. Single channel analyses were performed using pCLAMP 5.6 software (Axon Instruments, Foster City, CA) on current records low-pass filtered at 300 Hz through an 8-pole Bessel filter (902 LPF, Frequency Devices, Haverhill, MA) before acquisition using a Digidata 1200 interface (Axon Instruments). The actual number of functional ENaC channels (including those initially "silent") in each given experiment was determined by transiently activating them with the imposition of a hydrostatic gradient (Awayda et al., 1995; Ismailov et al., 1996a), thus excluding multiple channel incorporations from further experimentation and analyses. For illustration purposes, records were filtered at 100 Hz using a built-in digital filtering option of the pCLAMP software. Statistical significance of the quantitative differences between properties of single ENaCs in the presence and in the absence of CFTR were determined using a standard two-sample *t*-test for comparison of the means in two independent groups (Woolson, 1987).

## RESULTS

### Effects of truncation of cytoplasmic domains of ENaC subunits on kinetic properties of heterooligomeric amiloride-sensitive $Na^+$ channels

Figs. 1 through 3 depict representative current traces of single $\alpha\beta\gamma$-rENaCs made of the WT subunits and of the

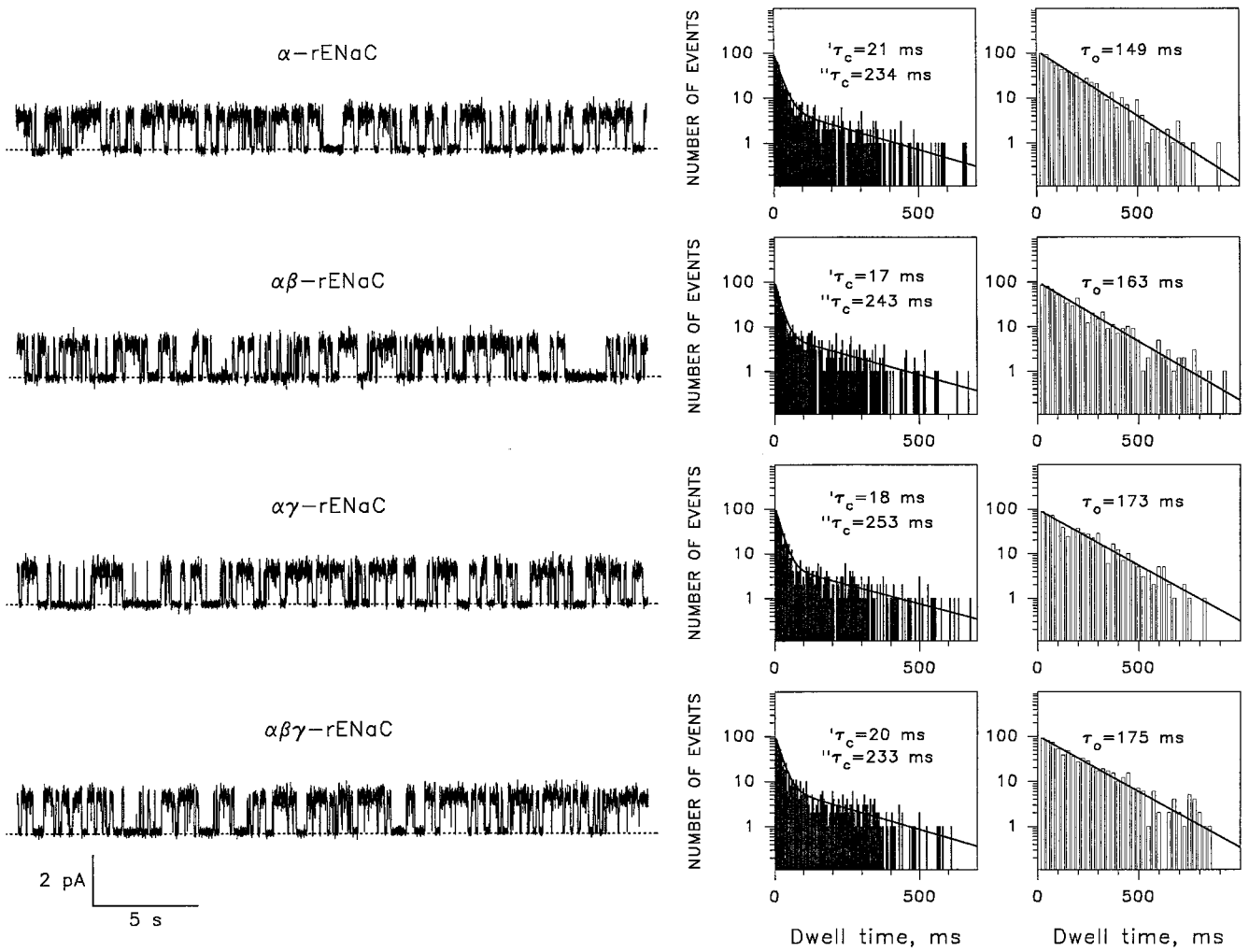


FIGURE 1 Single channel recordings of ENaCs made of different combinations of the WT subunits. Bilayers were bathed with symmetrical 100 mM NaMOPS solution complemented with 10 mM Tris (pH 7.4). The holding potential was +100 mV. For illustration purposes records representative of at least five separate experiments with each channel composition were filtered at 100 Hz. Representative dwell time histograms shown next to traces were constructed following events analysis performed using 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 using pCLAMP software and hardware. Event detection thresholds were 50% in amplitude and 3 ms in duration. The histograms shown were constructed from 633, 620, 627, and 681 events for $\alpha$, $\alpha\beta$, $\alpha\gamma$, and $\alpha\beta\gamma$, respectively. The bin widths in the closed and open time histograms were 5 and 25 ms, respectively. Closed and open time constants were determined from a double exponential ($y = a_1 \times \exp(-x/\tau_1) + a_2 \times \exp(-x/\tau_2)$), and a single exponential ($y = a \times \exp(-x/\tau)$) fit of the closed and open time histograms, respectively.

subunits with individual cytoplasmic domains truncated, and reconstituted into planar lipid bilayers. As can be seen in the figures, truncation of the amino-terminus of $\alpha$- (but not of the $\beta$- and/or $\gamma$-subunits), and truncation of the carboxy-termini of $\beta$- and/or $\gamma$-rENaC subunits (but not of the $\alpha$-subunit), have profound effects on kinetics of the channels (Figs. 2 and 3, respectively), as compared to the WT ENaCs (Fig. 1). The analyses of times spent by the channels in their open and closed states (see dwell time histograms next to traces) help to resolve the quantitative differences in their kinetic properties. Consistent with that reported previously (Ismailov et al., 1999), kinetics of the WT $\alpha\beta\gamma$-rENaCs in bilayers were characterized with double exponentially distributed closed times ($\tau_c' = 21 \pm 5$ ms, $\tau_c'' = 264 \pm 39$ ms), and single exponentially distributed open time ($\tau_o = 153 \pm 39$ ms). These properties were essentially indistinguishable from those of the channels made of the WT $\alpha$-rENaC, or of the heterodimeric $\alpha\beta$- and $\alpha\gamma$-channels (Table 1). The results of our experiments performed within this study demonstrate that all heterooligomeric channels in which $\alpha$-subunit was lacking its N-terminus display a more rapid kinetics, as compared to the WT channels, with the relatively short-lived (~40 ms) single exponentially distributed open and closed states (see Table 2 for the summary of numeric data), which was similar to gating of the homomultimeric channel made of the N-

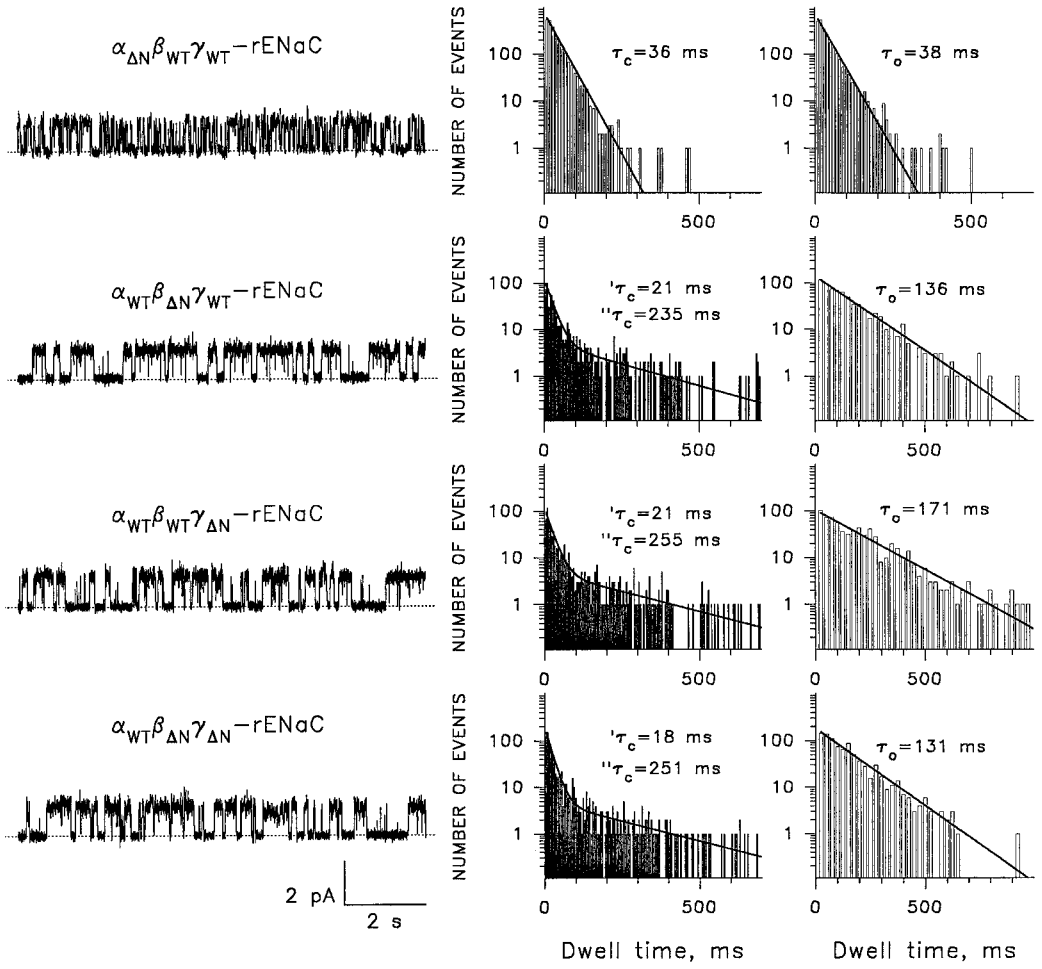


FIGURE 2 Single channel recordings of ENaCs made of the N-terminally truncated subunits. Recording conditions and single channel data handling were the same as described for Fig. 1. Closed and open time constants shown for the channels in which $\alpha$- subunit was N-terminally truncated were determined from a single exponential fit of the closed and open time histograms, respectively, binned with the 10 ms bin width. Numbers of events used for histograms were 2450, 699, 680, and 871 for $\alpha_{\Delta N}\beta_{WT}\gamma_{WT}$-, $\alpha_{WT}\beta_{\Delta N}\gamma_{WT}$-, $\alpha_{WT}\beta_{WT}\gamma_{\Delta N}$-, and $\alpha_{WT}\beta_{\Delta N}\gamma_{\Delta N}$-ENaCs, respectively.

terminally truncated $\alpha$-ENaC (Berdiev et al., 1998). Moreover, while the deletion of the amino-terminal domains in $\beta$- and/or $\gamma$-ENaCs did not appear to induce any significant changes in channel gating compared to the WT (see dwell time histograms next to respective traces in Fig. 2), truncation of this domain of the $\alpha$-subunit resulted in an "acceleration" of channel kinetics even in the heterooligomeric channels in which the C-termini of $\beta$- and/or $\gamma$-subunits were truncated: the distributions of times spent by the $\alpha_{\Delta N}\beta_{\Delta C}$-, $\alpha_{\Delta N}\gamma_{\Delta C}$-, $\alpha_{\Delta N}\beta_{WT}\gamma_{\Delta C}$-, $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{WT}$-, or $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{\Delta C}$-rENaC in their open or in the closed states could be fit to single exponentials (see Table 4 for numeric data), and were not much different from those of $\alpha_{\Delta N}$- or $\alpha_{\Delta N}\beta_{WT}\gamma_{WT}$-rENaC (Fig. 2 and Table 2). This finding was in contrast with a "continuously open" kinetics of the heterooligomeric channels made of the WT (or C-terminally truncated) $\alpha$-subunit and the C-terminally truncated $\beta$- and/or $\gamma$-ENaCs ($\alpha_{WT(\Delta C)}\beta_{\Delta C}$-, $\alpha_{WT(\Delta C)}\gamma_{\Delta C}$-, $\alpha_{WT(\Delta C)}\beta_{WT}\gamma_{\Delta C}$-, $\alpha_{WT(\Delta C)}\beta_{\Delta C}\gamma_{WT}$-, or $\alpha_{WT(\Delta C)}\beta_{\Delta C}\gamma_{\Delta C}$-rENaC), displaying a single short-lived closed time, with no apparent effect on the open time (Fig. 3 and Tables 3 and 4), as compared to the WT channels (Fig. 1). Based on these results, we conclude that the N-terminus of the $\alpha$-subunit is a major determinant of gating of both homooligomeric and heterooligomeric ENaCs.

## Effect of truncation of cytoplasmic domains in individual rENaC subunits on inhibition of heterooligomeric channels with CFTR

We have reported previously that the open probability of ENaCs in planar lipid bilayers was lower in the presence of CFTR in the membrane than in its absence (Ismailov, 1996b, 1997a). These findings were consistent with the hypothesis that CFTR inhibits ENaCs in normal airway

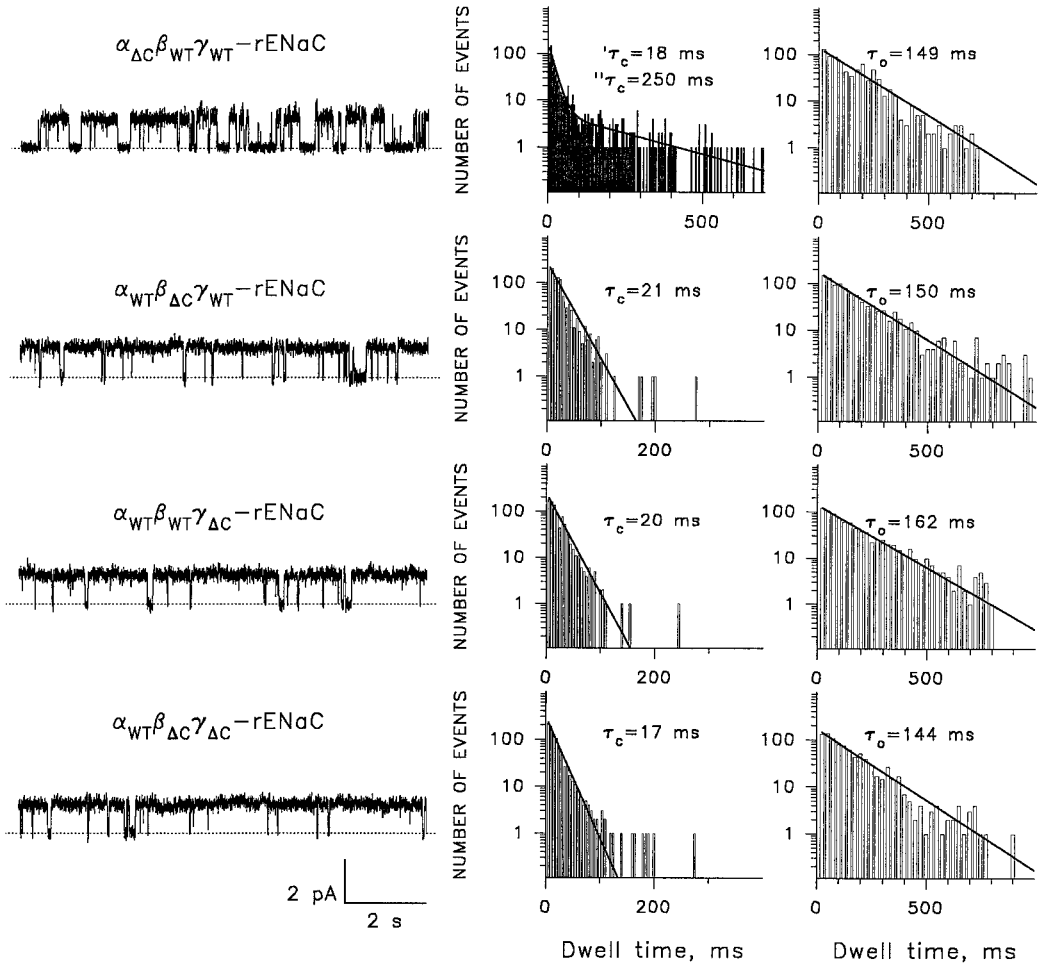


FIGURE 3 Single channel recordings of ENaCs made of the C-terminally truncated subunits. Recording conditions and single channel data handling were the same as described for Fig. 1. Closed time constants shown for the channels in which $\beta$- and/or $\gamma$-subunits were C-terminally truncated were determined from a single exponential fit of the closed time histograms, binned with the 5 ms bin width. Numbers of events used for histograms were 771, 991, 857, and 899 for $\alpha_{\Delta C}\beta_{WT}\gamma_{WT}$-, $\alpha_{WT}\beta_{\Delta C}\gamma_{WT}$-, $\alpha_{WT}\beta_{WT}\gamma_{\Delta C}$-, and $\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta C}$-ENaCs, respectively.

epithelia (Stutts et al., 1995, 1997; Ling et al., 1997; Mall et al., 1996; Kunzelmann et al., 1997; Briel et al., 1998; Chabot et al., 1999), and were considered as indicating that these two physiological entities directly interact with each other. However, no mechanistic interpretation of the phenomenon with respect to possible effect(s) of CFTR on ENaC gating was provided at the time, mostly due to the complex kinetic signature of the channel (see discussion of the relationship between "triple-barrel" model of ENaCs, Ismailov et al., 1996a, with single channels in Berdiev et al., 1998). In order to determine the functional significance of different structural domains in channel gating and in the mechanism of the CFTR-induced inhibition of ENaCs, we examined the effects of CFTR on single ENaCs made of the WT and/or N- and C-terminally truncated subunits.

**TABLE 1 Kinetic properties of the wild-type ENaCs in bilayers**

| | Dwell Time (M ± m, ms) | | | Number of Single Channel Events Fitted/Experiment |
|---|---|---|---|---|
| | $\tau_o$ | $\tau'_c$ | $\tau''_c$ | |
| $\alpha$ ($n$ = 5) | 160 ± 30 | 20 ± 7 | 245 ± 50 | ≥611 |
| $\alpha\beta$ ($n$ = 8) | 142 ± 41 | 17 ± 6 | 259 ± 42 | ≥601 |
| $\alpha\gamma$ ($n$ = 6) | 140 ± 36 | 19 ± 8 | 248 ± 45 | ≥624 |
| $\alpha\beta\gamma$ ($n$ = 6) | 153 ± 39 | 21 ± 5 | 264 ± 39 | ≥657 |

Fig. 4 *A* displays representative single channel recordings of the individual 13 pS conduction elements of $\alpha$- and $\alpha\beta\gamma$-ENaC in the presence of immunopurified bovine tracheal CFTR co-reconstituted into planar lipid bilayers, and bathed with $Cl^-$-free solutions. The experimental design was basically the same as described previously (Ismailov et al., 1996b, 1997a). Namely, following the assessment of $Na^+$ currents through single ENaCs, the presence and the orientation of CFTR channels in the membrane were determined by activating them with a catalytic subunit of protein kinase A in the bathing solution supplemented with 100 mM

**TABLE 2 Effect of truncation of amino-terminal cytoplasmic domains in individual rENaC subunits on kinetic properties of homo and heterooligomeric channels**

| | Dwell Time (M ± m, ms) | | | Number of Single Channel Events Fitted/Experiment |
|---|---|---|---|---|
| | $\tau_o$ | $\tau'_c$ | $\tau''_c$ | |
| *Homooligomer* | | | | |
| $\alpha_{\Delta N}$ ($n = 4$) | 30 ± 10 | 34 ± 11 | Not Apparent | ≥2815 |
| *Heterodimers* | | | | |
| $\alpha_{\Delta N}\beta$ ($n = 6$) | 33 ± 12 | 39 ± 10 | Not Apparent | ≥3001 |
| $\alpha_{\Delta N}\gamma$ ($n = 5$) | 40 ± 11 | 32 ± 9 | Not Apparent | ≥2726 |
| $\alpha\beta_{\Delta N}$ ($n = 5$) | 155 ± 42 | 16 ± 5 | 232 ± 40 | ≥911 |
| $\alpha\gamma_{\Delta N}$ ($n = 7$) | 140 ± 33 | 17 ± 6 | 250 ± 40 | ≥748 |
| *Heterotrimers* | | | | |
| $\alpha_{\Delta N}\beta\gamma$ ($n = 5$) | 42 ± 10 | 31 ± 11 | Not Apparent | ≥2415 |
| $\alpha\beta_{\Delta N}\gamma$ ($n = 6$) | 169 ± 32 | 20 ± 6 | 251 ± 39 | ≥681 |
| $\alpha\beta\gamma_{\Delta N}$ ($n = 7$) | 173 ± 38 | 19 ± 7 | 274 ± 37 | ≥644 |
| $\alpha_{\Delta N}\beta_{\Delta N}\gamma$ ($n = 7$) | 31 ± 8 | 39 ± 10 | Not Apparent | ≥2557 |
| $\alpha_{\Delta N}\beta\gamma_{\Delta N}$ ($n = 4$) | 40 ± 12 | 32 ± 9 | Not Apparent | ≥2345 |
| $\alpha\beta_{\Delta N}\gamma_{\Delta N}$ ($n = 5$) | 151 ± 33 | 17 ± 6 | 263 ± 44 | ≥843 |
| $\alpha_{\Delta N}\beta_{\Delta N}\gamma_{\Delta N}$ ($n = 5$) | 32 ± 11 | 41 ± 10 | Not Apparent | ≥2863 |

CsCl and 100 $\mu$M Mg-ATP. In addition, each experiment was concluded with the identification of both ENaC and CFTR by testing their sensitivities to amiloride and diphenylamine-2-carboxylic acid (DPC), respectively. As can be seen in the figure, in the presence of CFTR the time spent by $\alpha\beta\gamma$-, $\alpha\beta$-, or $\alpha\gamma$-rENaC in their closed states can be fit to two exponentials, one with a constant of ~25 ms (which is not much different from that of the channels in the absence of CFTR, or that of the $\alpha$-rENaC, with or without CFTR), and another with a constant of ~350 ms (which is longer then that of that the channels in the absence of CFTR, or that of $\alpha$-rENaC, with or without CFTR). No apparent effect of CFTR on the time spent by ENaCs in their open state was found. These data suggest that CFTR can modulate gating of heterooligomeric ENaCs by lengthening their closed time. This can account for the decrease in the open probability of single $\alpha\beta\gamma$-, $\alpha\beta$-, or $\alpha\gamma$-rENaC in the presence of CFTR, as compared with the channels in the absence of CFTR, or with the $\alpha$-rENaC in the presence of CFTR (Fig. 4 *B*).

**TABLE 3 Effect of truncation of carboxy-terminal cytoplasmic domains in individual rENaC subunits on kinetic properties of homo and heterooligomeric channels**

| | Dwell Time (M ± m, ms) | | | Number of Single Channel Events Fitted/Experiment |
|---|---|---|---|---|
| | $\tau_o$ | $\tau'_c$ | $\tau''_c$ | |
| *Homooligomer* | | | | |
| $\alpha_{\Delta C}$ ($n = 5$) | 134 ± 40 | 16 ± 6 | 234 ± 33 | ≥715 |
| *Heterodimers* | | | | |
| $\alpha_{\Delta C}\beta$ ($n = 7$) | 151 ± 33 | 17 ± 5 | 266 ± 48 | ≥844 |
| $\alpha_{\Delta C}\gamma$ ($n = 5$) | 171 ± 43 | 22 ± 7 | 271 ± 46 | ≥875 |
| $\alpha\beta_{\Delta C}$ ($n = 4$) | 149 ± 36 | 17 ± 6 | Not Apparent | ≥915 |
| $\alpha\gamma_{\Delta C}$ ($n = 4$) | 166 ± 38 | 20 ± 8 | Not Apparent | ≥796 |
| *Heterotrimers* | | | | |
| $\alpha_{\Delta C}\beta\gamma$ ($n = 4$) | 165 ± 46 | 20 ± 6 | 239 ± 34 | ≥750 |
| $\alpha\beta_{\Delta C}\gamma$ ($n = 6$) | 141 ± 34 | 18 ± 7 | Not Apparent | ≥942 |
| $\alpha\beta\gamma_{\Delta C}$ ($n = 6$) | 132 ± 42 | 21 ± 6 | Not Apparent | ≥847 |
| $\alpha_{\Delta C}\beta_{\Delta C}\gamma$ ($n = 5$) | 133 ± 36 | 20 ± 8 | Not Apparent | ≥902 |
| $\alpha_{\Delta C}\beta\gamma_{\Delta C}$ ($n = 8$) | 148 ± 31 | 17 ± 7 | Not Apparent | ≥827 |
| $\alpha\beta_{\Delta C}\gamma_{\Delta C}$ ($n = 5$) | 161 ± 42 | 22 ± 9 | Not Apparent | ≥867 |
| $\alpha_{\Delta C}\beta_{\Delta C}\gamma_{\Delta C}$ ($n = 4$) | 157 ± 44 | 19 ± 8 | Not Apparent | ≥798 |

The effects of truncation of individual cytoplasmic domains of ENaC subunits on inhibition of these channels by CFTR were studied next. The results of these experiments indicate that kinetic properties of all heterooligomeric channels containing either the amino-terminally truncated (see top trace in Fig. 5 *A*), or the carboxy-terminally truncated (see top trace in Fig. 6 *A*), $\alpha$-rENaC in the presence of CFTR in the membrane were not significantly different from those in the absence of CFTR. The same was true for open probability of these channels (see bar graphs in Figs. 5 *B* and 6 *B*). Considering the fact that, as it was shown in the preceding paragraph, CFTR inhibits amiloride-sensitive $Na^+$ channels, at least in part, by altering their gating, the finding that truncation of the N-terminal domain of $\alpha$-subunit completely abolishes the effect of CFTR on ENaCs is consistent with the aforementioned conclusion that this domain plays a major role in determining kinetics of these channels. The lack of an effect of CFTR on the channels containing the C-terminally $\alpha$-ENaC (including heterooligomeric combinations of $\alpha_{\Delta C}$ with the WT $\beta$- and $\gamma$-), however, can be explained by the finding of Kunzelmann et al. (1997) that CFTR interacts directly with this, but not with any other domain of ENaCs.

We have also shown previously and here that CFTR exerts its inhibitory effect only on heterooligomeric ENaCs, in which $\beta$- and/or $\gamma$- subunits are important for modulation of the channel gating. Then, considering the fact that CFTR affects ENaC gating by modulating closed time, the kinetic parameter that is affected by truncation of the carboxy-termini of these two subunits, one possible scenario is that CFTR interferes with channel gating via these domains. If so, in the absence of the C-termini of $\beta$- and $\gamma$-ENaC, CFTR should fail to inhibit amiloride-sensitive channels. Measurements of the elevated nasal transepithelial potential difference in Liddle's disease (genetically linked to premature stop-codon mutations in C-termini of $\beta$- and/or $\gamma$-ENaCs; Shimkets et al., 1994; Hansson et al., 1995a, b) patients by Baker et al. (1998) support this line of considerations. The results of our experiments in bilayers suggest that, in contrast to these predictions, CFTR effectively downregulated heterooligomeric $\alpha_{WT}\beta_{\Delta C}$-, $\alpha_{WT}\gamma_{\Delta C}$-, $\alpha_{WT}\beta_{\Delta C}\gamma_{WT}$-, $\alpha_{WT}\beta_{WT}\gamma_{\Delta C}$-, and $\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta C}$-ENaCs (see respective traces in Fig. 6 *A* and, for the summary of the open probability data, Fig. 6 *B*), despite the fact that the C-terminal domains in $\beta$- and/or $\gamma$-subunits were eliminated by trun-

**TABLE 4 Effect of truncation of two or three different cytoplasmic domains in individual rENaC subunits on kinetic properties of heterotrimeric channels**

| | Dwell Time (M ± m, ms) | | | Number of Single Channel Events Fitted/Experiment |
|---|---|---|---|---|
| | $\tau_o$ | $\tau_c'$ | $\tau_c''$ | |
| | | *α- and β-truncation* | | |
| $\alpha_{\Delta C}\beta_{\Delta N}$ ($n = 6$) | 151 ± 38 | 18 ± 7 | 243 ± 51 | ≥898 |
| $\alpha_{\Delta N}\beta_{\Delta C}$ ($n = 8$) | 37 ± 8 | 33 ± 10 | Not Apparent | ≥3279 |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma$ ($n = 5$) | 145 ± 40 | 21 ± 6 | 255 ± 40 | ≥760 |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma$ ($n = 4$) | 38 ± 10 | 36 ± 11 | Not Apparent | ≥2490 |
| | | *α- and γ-truncations* | | |
| $\alpha_{\Delta C}\gamma_{\Delta N}$ ($n = 4$) | 156 ± 43 | 20 ± 6 | 261 ± 38 | ≥657 |
| $\alpha_{\Delta N}\gamma_{\Delta C}$ ($n = 5$) | 39 ± 11 | 36 ± 9 | Not Apparent | ≥2605 |
| $\alpha_{\Delta C}\beta\gamma_{\Delta N}$ ($n = 5$) | 153 ± 40 | 18 ± 6 | 269 ± 50 | ≥840 |
| $\alpha_{\Delta N}\beta\gamma_{\Delta C}$ ($n = 6$) | 35 ± 11 | 29 ± 8 | Not Apparent | ≥3030 |
| | | *β- and γ-truncations* | | |
| $\alpha\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 5$) | 173 ± 40 | 16 ± 7 | Not Apparent | ≥782 |
| $\alpha\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 6$) | 164 ± 31 | 19 ± 7 | Not Apparent | ≥829 |
| | | *α-, β-, and γ-truncations* | | |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{\Delta C}$ ($n = 5$) | 34 ± 10 | 37 ± 9 | Not Apparent | ≥3210 |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 6$) | 138 ± 31 | 15 ± 6 | Not Apparent | ≥822 |
| $\alpha_{\Delta C}\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 4$) | 167 ± 42 | 18 ± 7 | Not Apparent | ≥914 |
| $\alpha_{\Delta N}\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 5$) | 30 ± 11 | 37 ± 10 | Not Apparent | ≥2902 |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 7$) | 41 ± 9 | 36 ± 11 | Not Apparent | ≥2445 |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma_{\Delta N}$ ($n = 5$) | 139 ± 35 | 21 ± 8 | 270 ± 48 | ≥770 |

cation and the channels were continuously active in the absence of CFTR (Fig. 3). These findings are consistent with the recent report of Hopf et al. (1999) that, in the presence of CFTR, macroscopic amiloride-sensitive $Na^+$ currents induced in *Xenopus* oocytes by ENaCs in which β- and γ-subunits were C-terminally truncated were lower than those in its absence. Moreover, our data suggest that, similar to that in the WT $\alpha\beta\gamma$-ENaCs, the inhibitory effect of CFTR on all of these channels was manifested in a lengthening of the longer component of their closed time (see representative analyses of single channel kinetics shown next to $\alpha_{WT}\beta_{\Delta C}\gamma_{WT}$, $\alpha_{WT}\beta_{WT}\gamma_{\Delta C}$, and $\alpha\beta_{\Delta C}\gamma_{\Delta C}$ traces in Fig. 6 *A*).

With regard to the N-terminal tails of β- and γ-subunits, which were also hypothesized to play role in gating of heterooligomeric ENaCs (Gründer et al., 1997), truncation of these domains abolished inhibitory effects of CFTR on the heterodimeric $\alpha_{WT}\beta_{\Delta N}$-, and $\alpha_{WT}\gamma_{\Delta N}$-, but not on the heterotrimeric $\alpha_{WT}\beta_{\Delta N}\gamma_{WT}$- or $\alpha_{WT}\beta_{WT}\gamma_{\Delta N}$-ENaCs. That is, in the presence of CFTR, both gating and open probability of the $\alpha_{WT}\beta_{\Delta N}$- and $\alpha_{WT}\gamma_{\Delta N}$-ENaCs were not significantly different from those in its absence, whereas $\alpha_{WT}\beta_{\Delta N}\gamma_{WT}$- and $\alpha_{WT}\beta_{WT}\gamma_{\Delta N}$-ENaCs displayed both the lengthening of their closed time and a decrease in $P_o$. The latter was also true for the continuously active $\alpha_{WT}\beta_{\Delta N}\gamma_{\Delta C}$- and $\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta N}$-ENaCs (Table 5). However, the inhibitory effect of CFTR on ENaCs was completely abolished when the heterotrimeric channel contained both N-terminally truncated β- and γ-mutants (compare the fourth trace in Fig. 5 *A* with the second and third traces in this figure; the summary of the open probability data is shown in Fig. 5 *B*). Although the mechanism underlying these phenomena is not entirely clear at this point, these findings are especially intriguing, considering the lack of any apparent effect of truncation of these domains on kinetic properties of ENaCs as compared to the WT channels (see Figs. 1 and 2).

## DISCUSSION

The issue that should be emphasized first when interpreting the results of the present study searching for the specific structural domain(s) that act as a "gate" of the amiloride-sensitive $Na^+$ channels is related to the heterooligomeric structure of this channel. The fact is that the α-, the first of the three cloned ENaC subunits, is sufficient to form functional amiloride-sensitive $Na^+$ channels in *Xenopus* oocytes, lipid bilayers, and LM($TK^-$) cells (Canessa et al., 1993, 1994; Lingueglia et al., 1993; Ismailov et al., 1996a; Kizer et al., 1997; Berdiev et al., 1998), whereas under no circumstances do β- and/or γ-ENaCs conduct any current. Yet, these subunits appear to substitute two α subunits in the otherwise tetrameric complex (compare Firsov et al., 1998 and Kosari et al., 1998 with Berdiev et al., 1998 and Coscoy et al., 1998). Besides participation of β- and γ-ENaCs in cooperative assembly, trafficking, and surface expression of amiloride-sensitive channels in cells (Canessa et al., 1993, 1994; Awayda et al., 1997; Valentijn et al., 1998; Cheng et al., 1998; Prince and Welsh, 1998, 1999), the roles of these subunits in channel function, particularly with respect to its gating, remain largely unknown. Besides that, the issue that should be kept in mind when devising the relationship(s)

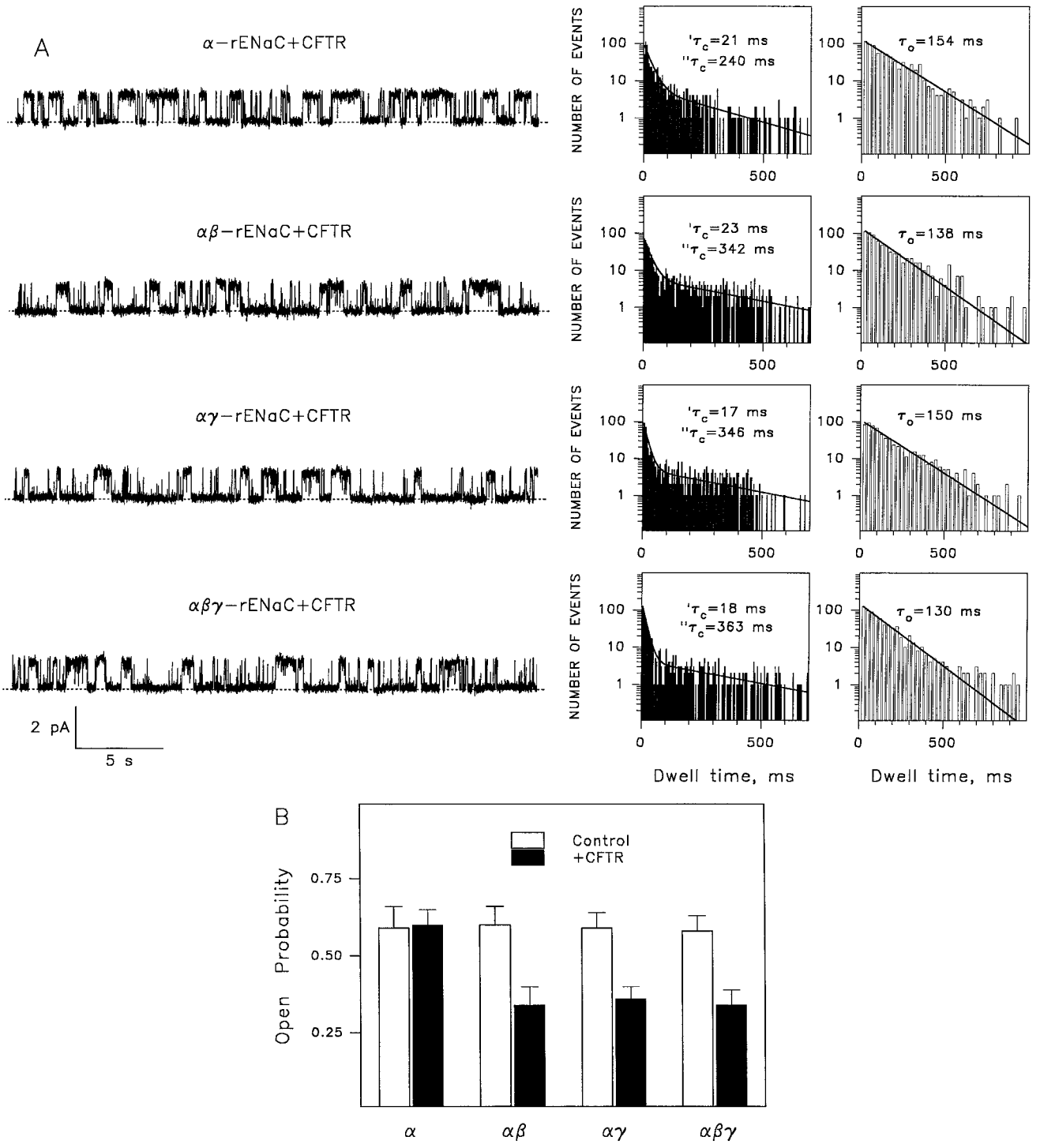


FIGURE 4 Effects of CFTR on single homooligomeric ($\alpha$-) and heterooligomeric ($\alpha\beta$-, $\alpha\gamma$- and $\alpha\beta\gamma$-) ENaCs. Conditions for recording and single channel kinetic analyses shown in (*A*) were the same as described in Fig. 1. Not shown in the figure, the presence and the orientation of CFTR channels in the membrane were determined in each experiment following the assessment of $Na^+$ currents through single ENaCs by activating them with a catalytic subunit of protein kinase A in the bathing solution supplemented with 100 mM CsCl and 100 $\mu$M Mg-ATP. In addition, the identity of ENaC and CFTR was confirmed in the end of each experiment by testing their sensitivity to amiloride and DPC, respectively. Records shown are representative of at least six separate co-incorporations/co-orientations of CFTR with each channel composed of ENaC subunits, as indicated in the figure. Closed and open time constants were determined from a double exponential and a single exponential fit of the closed and open time histograms, respectively, as described in Fig. 1. The bin widths in the closed and open time histograms were 5 and 25 ms, respectively. Numbers of events used for histograms were 742, 718, 626, and 716 for $\alpha$-, $\alpha\beta$-, $\alpha\gamma$-, and $\alpha\beta\gamma$-ENaCs, respectively. (*B*) A summary bar graph of the effects of CFTR on open probability of single homooligomeric ($\alpha$-) and heterooligomeric ($\alpha\beta$-, $\alpha\gamma$-, and $\alpha\beta\gamma$-) ENaCs.

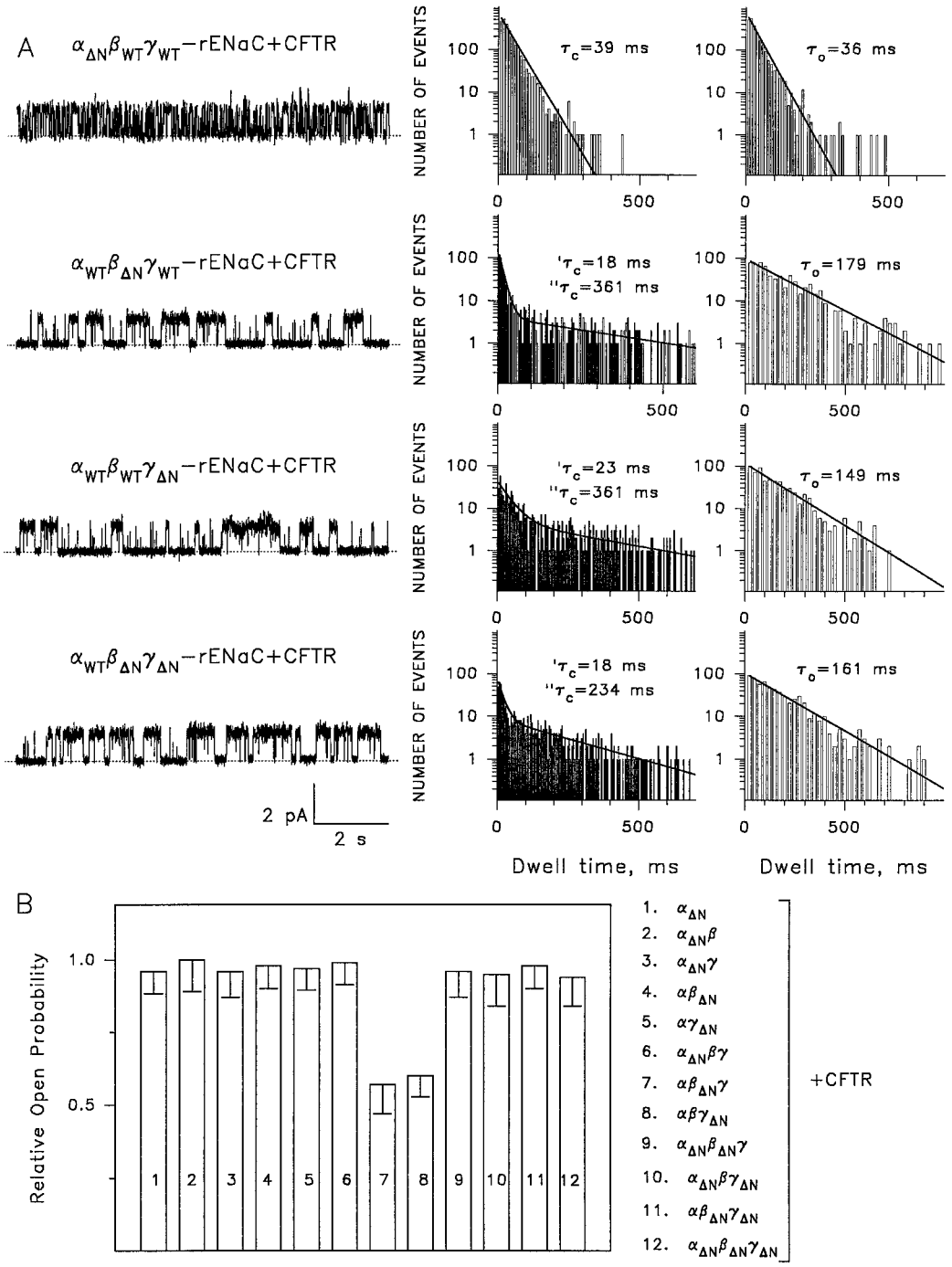


FIGURE 5 Effects of truncation of amino-terminal cytoplasmic domains of individual ENaC subunits on inhibition of heterooligomeric amiloride-sensitive $Na^+$ channels by CFTR. (*A*) Recording conditions and single channel kinetic analyses were the same as described in Fig. 1. Protocols for identification of co-incorporation of ENaC and CFTR were the same as described in Fig. 4. Records shown were filtered at 100 Hz and represent at least four separate co-incorporations/co-orientations of CFTR with each channel composed of ENaC subunits, as indicated in the figure. The bin widths in the closed and open time histograms for the channels in which $\alpha$-subunits were wild type were 5 and 25 ms, respectively. Closed and open time constants were determined from a double-exponential and single-exponential fit of the closed and open time histograms, respectively. Closed and open time constants shown for the channels in which $\alpha$-subunits were N-terminally truncated were determined from a single-exponential fit of closed and open time histograms, respectively, binned with the 10 ms bin width. Numbers of events used for construction of the histograms shown were 2385, 643, 632, and 622 for $\alpha_{\Delta N}\beta_{WT}\gamma_{WT}$-, $\alpha_{WT}\beta_{\Delta N}\gamma_{WT}$-, $\alpha_{WT}\beta_{WT}\gamma_{\Delta N}$-, and $\alpha_{WT}\beta_{\Delta N}\gamma_{\Delta N}$-ENaCs, respectively. (*B*) A summary bar graph of the effects of CFTR on open probability of single homooligomeric and heterooligomeric ENaCs with individual cytoplasmic domains truncated.

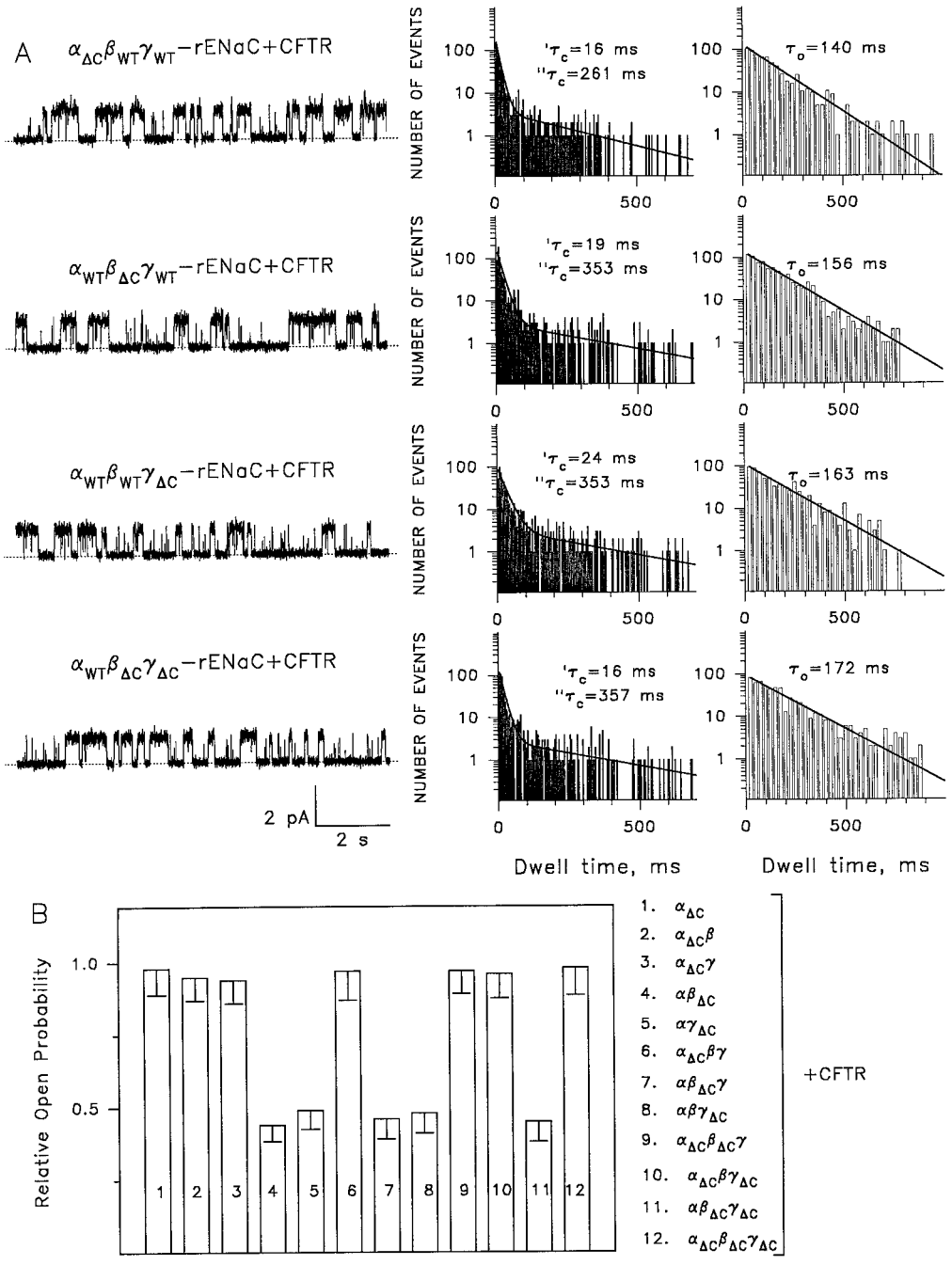


FIGURE 6 Effects of truncation of carboxy-terminal cytoplasmic domains of individual ENaC subunits on inhibition of heterooligomeric amiloride-sensitive $Na^+$ channels by CFTR. (*A*) Recording conditions and single channel kinetic analyses were the same as described in Fig. 1. Protocols for identification of co-incorporation of ENaC and CFTR were the same as described in Fig. 4. Records shown represent at least four separate co-incorporations/co-orientations of CFTR with each channel composed of ENaC subunits, as indicated in the figure. The bin widths in the closed and open time histograms for the channels in which $\alpha$- subunits were WT or C-terminally truncated were 5 and 25 ms, respectively. Closed and open time constants were determined from a double-exponential and single-exponential fit of the closed and open time histograms, respectively. Numbers of events used for the histogram construction were 676, 794, 682, and 617 for $\alpha_{\Delta C}\beta_{WT}\gamma_{WT}$-, $\alpha_{WT}\beta_{\Delta C}\gamma_{WT}$-, $\alpha_{WT}\beta_{WT}\gamma_{\Delta C}$-, and $\alpha_{WT}\beta_{\Delta C}\gamma_{\Delta C}$-ENaCs, respectively. (*B*) A summary bar graph of the effects of CFTR on open probability of single homooligomeric and heterooligomeric ENaCs with individual cytoplasmic domains truncated.

between structure and the function of rather complex heterooligomeric entities, such as ENaCs, is that the changes in gating of an ion channel induced by mutations may not be due to directly placing or removing physical barrier(s) along the length of the conduction pathway, but may result from allosteric effects. All these circumstances necessitate the

**TABLE 5 Effect of truncation of two or three different cytoplasmic domains in individual rENaC subunits on inhibition of heterotrimeric channels by CFTR**

| | Effect of CFTR |
|---|---|
| *α- and β-truncation* | |
| $\alpha_{\Delta C}\beta_{\Delta N}$ ($n = 7$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\beta_{\Delta C}$ ($n = 5$) | No ($P > 0.5$) |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma$ ($n = 4$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma$ ($n = 6$) | No ($P > 0.5$) |
| *α- and γ-truncations* | |
| $\alpha_{\Delta C}\gamma_{\Delta N}$ ($n = 4$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\gamma_{\Delta C}$ ($n = 6$) | No ($P > 0.5$) |
| $\alpha_{\Delta C}\beta\gamma_{\Delta N}$ ($n = 6$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\beta\gamma_{\Delta C}$ ($n = 5$) | No ($P > 0.5$) |
| *β- and γ-truncations* | |
| $\alpha\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 7$) | Yes ($P < 0.001$) |
| $\alpha\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 5$) | Yes ($P < 0.001$) |
| *α-, β-, and γ-truncations* | |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{\Delta C}$ ($n = 4$) | No ($P > 0.5$) |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 6$) | No ($P > 0.5$) |
| $\alpha_{\Delta C}\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 5$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\beta_{\Delta N}\gamma_{\Delta C}$ ($n = 7$) | No ($P > 0.5$) |
| $\alpha_{\Delta N}\beta_{\Delta C}\gamma_{\Delta N}$ ($n = 5$) | No ($P > 0.5$) |
| $\alpha_{\Delta C}\beta_{\Delta N}\gamma_{\Delta N}$ ($n = 8$) | No ($P > 0.5$) |

discussion of the results of this study in terms of the relationship(s) between the structure of *each* of these newly identified gene products, and the biophysical properties of *each* heterooligomeric entity formed.

We have previously hypothesized that there must be at least two separate gating processes: one inherent to α-ENaC and another superimposed on the first one due to formation of a heterooligomer of α- with β- and γ-subunits (Ismailov et al., 1999). Besides the aforementioned evidence that the α-subunit is the only one of three capable of forming a channel (which, by definition, should have a gate of its own), this hypothesis was based on the following of considerations. First, although the overall kinetic properties of αβγ-, αβ-, or αγ-ENaC did not differ significantly from those of α-ENaC (Ismailov et al., 1999), truncation of the carboxy-termini of β- and γ-ENaC has been shown to affect gating of the heterooligomeric channels, converting them into continuously active ones (Schild et al., 1995; Firsov et al., 1996; Ismailov et al., 1999) characteristic of Liddle's genetic hypertension disease. Second, synthetic peptides with the sequences comprising the distal 30-residue domains of the carboxy termini of the β- and γ-hENaC, when added to the solution bathing the presumptive cytoplasmic face of the $\alpha\beta_{\Delta C}\gamma_{\Delta C}$-rENaCs, decreased open probability of these continuously active channels, once again, by changing their gating (Ismailov et al., 1999). Third, the effects of the inhibitory β- and γ-peptides were synergistic: they "locked" $\alpha\beta_{\Delta C}\gamma_{\Delta C}$-rENaCs in the closed state more effectively when applied together than separately from each other, but did not exert any action (either separately or together) on the WT αβγ-ENaCs. Altogether, these results were interpreted as suggestive that cytoplasmic domains of *all* three subunits participate in determining kinetic properties of heterooligomeric ENaCs, whereas the α-channel has its own fully competent gate.

The amino-terminus of the α-subunit was proposed as a candidate domain for the role of such a gate based on the results of electrophysiological experiments of Gründer et al. (1997) with heterooligomeric ENaCs bearing a substitution mutation in this domain. These channels exhibited a reduced macroscopic amiloride-sensitive current in oocytes (as compared with the WT ENaCs), which was attributed, at least in part, to defective channel gating. Consistent with this hypothesis, homooligomeric and all heterooligomeric channels in which α-ENaC was N-terminally truncated observed in our experiments in bilayers performed previously (Berdiev et al., 1998), and within present study, had quite different kinetic properties compared to those of the WT α-, αβ-, αγ-, and αβγ-ENaCs. Truncation of the N-termini of β- and γ-subunits, however, did not have any apparent effect on gating of single αβγ-rENaCs in bilayers, which was also consistent with the finding of Gründer et al. (1997) that specific mutation of the N-terminus of the β-ENaC subunit identified by genetic-linkage analyses in patients affected with the salt-wasting syndrome pseudohypoaldosteronism type 1, namely, G37S (Chang et al., 1996) did not induce any changes in gating pattern of the channel expressed in *Xenopus* oocytes together with the WT α- and γ-ENaCs. However, unlike that in the experiments of Gründer et al. (1997) in *Xenopus* oocytes, in bilayers none of the channels formed by the N-terminally truncated subunits (including those with altered gating) exhibited any evident change in open probability (both the open and the closed time shorten, with the fraction of time spent by the channel in its open state essentially unchanged), that could account for the "loss-of-function" defect, which is a characteristic feature of the PHA-1 phenotype. This can be explained by the fact that the G95S mutation may not accurately reflect the pathophysiological mechanism(s) triggered by the frame-shift mutation at the nucleotides corresponding to the isoleucine residue in position 68 of the α-hENaC sequence (I93 in α-rENaC) actually taking place in several kindreds representing this disease (Chang et al., 1996). As a result of this frame-shift, the α-ENaC polypeptide should be only 144 residues in length, bearing no similarity to the normal ENaC from amino acid 68 to 144, where translation is terminated by a premature stop codon. Then, a complete loss of channel function in PHA-1 may be due to insufficiency of the pore-forming subunit. Nonetheless, the observation that the effect of N-terminal truncation in α-ENaC on kinetic properties of channels in bilayers prevailed in all studied combinations of this subunit with β- and/or γ- (including those with the C-terminally truncated β- and γ-), taken together with our previous finding that the "accelerated" gating (which is a feature of the $\alpha_{\Delta N}$-mutant)

dominated the phenotype of the channel in $\alpha_{WT}/\alpha_{\Delta N}$-rENaC mixtures with no graded changes observed (Berdiev et al., 1998), suggests that this domain is important for determining kinetic properties of both homoligomeric and heterooligomeric ENaCs. This, however, is not to ascribe the gate function of ENaCs solely to the N-terminus of the $\alpha$-subunit: even with this entire domain deleted, both the homooligomeric and the heterooligomeric ENaCs still open and close in a well-discernible manner.

The effects of CFTR on ENaC gating both parallel and contrast the inhibition of ENaCs with the peptides comprising the most distal 30 residue stretches of the C-termini of $\beta$- and $\gamma$-subunits (Ismailov et al., 1999). Particularly, these peptides were shown to restore the longer component of the closed time of $\alpha\beta_{\Delta C}\gamma_{\Delta C}$-ENaCs, which virtually disappeared from the kinetics of these continuously active channels due to carboxy-terminal truncation of $\beta$- and/or $\gamma$-subunits (Ismailov et al., 1999). Likewise, the inhibitory effect of CFTR on ENaCs also manifested in a lengthening of the longer component of their closed time. Furthermore, the presence of CFTR in the membrane did not have any effect on the channels formed by $\alpha$-rENaC alone, but inhibited both heterodimeric and heterotrimeric combinations of this subunit with $\beta$- and/or $\gamma$-ENaCs. The peptides, unlike CFTR, had no effect on the WT $\alpha\beta$-, $\alpha\gamma$-, or $\alpha\beta\gamma$-ENaCs. All these results are consistent with the idea of an integrated gating mechanism between all three (or two) ENaC subunits in a heterooligomer, which appears to be affected in the presence of the CFTR in the membrane. However, our results demonstrate that CFTR can induce similar effects (i.e., lengthening of the closed time) in the heterodimeric and heterotrimeric channels lacking C-termini of one or even both $\beta$- and $\gamma$-subunits.

These observations, which are consistent with the results of a recent study by Hopf et al. (1999), indicating that macroscopic amiloride-sensitive $Na^+$ currents induced in *Xenopus* oocytes by ENaCs in which $\beta$- and $\gamma$-subunits were C-terminally truncated were lower in the presence of CFTR than those in its absence, deserve separate discussion with respect to their possible relation to interactions between different structural domains of ENaC subunits, and to the role of these interactions in channel gating. First, taken together with the aforementioned finding, the "accelerated" channel phenotype characteristic for the N-terminally truncated $\alpha$-ENaC prevailed in all studied heterodimeric and heterotrimeric combinations of this subunit with $\beta$- and/or $\gamma$- (including those with the C-terminally truncated $\beta$- and $\gamma$-); these findings may suggest that, although the carboxy-terminal domains of $\beta$- and $\gamma$-ENaC subunits may play a role in modulation of the kinetics of heterooligomeric channels, the mechanism underlying this modulation does not completely override the gating mechanism, formed supposedly by the N-termini of the $\alpha$-subunits. Lack of inhibition by CFTR of *all* channels in which $\alpha$-subunit was N-terminally truncated supports this hypothesis. Second, if the $\alpha$-channel has a gate of its own, $\beta$- and $\gamma$- subunits (but not their C-termini) are required for CFTR to modulate its function. This consideration raises a question about the mechanism of how these domains mediate modulatory effects of $\beta$- and $\gamma$-ENaCs on channel gating. A possibility of direct physical interaction between CFTR and ENaCs taking place in the C-terminus of $\alpha$-subunits was suggested by the results of the yeast two hybrid analyses of Kunzelmann et al. (1997). Our observation that truncation of the C-terminal tail of $\alpha$-ENaC abolished inhibition of heterooligomeric channels by CFTR supports this hypothesis, although we can not totally exclude a possibility that some minor contaminant accessory proteins co-immunopurified with CFTR contribute to our findings in planar lipid bilayer reconstitution experiments. The latter reservation does not affect the following line of considerations aimed to mechanistically explain our data. Namely, it seems plausible to hypothesize that CFTR (with or without accessory proteins) and the $\beta$- and $\gamma$-peptides may interact with a site in the $\alpha$-C-terminus (which becomes accessible only in a heterooligomeric channel), thereby exerting their effects on channel gating determined largely by $\alpha$ $NH_2$-terminus.

The roles of the N-terminal domains of $\beta$- and $\gamma$-ENaC in channel gating are less clear. Our data demonstrate that truncation of one or both these domains (while keeping the WT $\alpha$-) did not induce any apparent changes in gating of heterooligomeric channels, as compared to their WT counterparts. Yet, it abolished the inhibitory effect of CFTR on $\alpha_{WT}\beta_{\Delta N}$- and $\alpha_{WT}\gamma_{\Delta N}$-heterodimers and $\alpha_{WT}\beta_{\Delta N}\gamma_{\Delta N}$ heterotrimers. The heterotrimers with the N-terminal tail of one of these two subunits ($\beta$- or $\gamma$-) truncated (while keeping another one, i.e., $\gamma$- or $\beta$-, respectively, WT) were not any different from the WT $\alpha\beta\gamma$-ENaCs regarding these effects of CFTR, namely, CFTR affected both the gating and the open probability of these channels. The heterotrimers with one subunit ($\beta$- or $\gamma$-) lacking its carboxy-terminus and another subunit ($\gamma$- or $\beta$-, respectively) lacking its amino-terminus could be inhibited by CFTR as well. A possibility that may help to understand these data is based on the conclusion of Adams et al. (1997) that the N-terminally truncated $\gamma$-ENaC assembles into an "unstable complex with the full length subunits," and that $\gamma$-N-terminus is a site of heterooligomeric assembly of this channel. This conclusion was based on the findings of the authors that this domain 1) was sufficient, but not required for co-immunoprecipitation of $\alpha$ENaC from the lysates of transfected COS-7 cells; 2) was sufficient to inhibit amiloride-sensitive currents induced in *Xenopus* oocytes by expression of the WT $\alpha\beta\gamma$-ENaCs; and that 3) its truncation dramatically reduced the magnitude of amiloride-sensitive currents and cell surface expression of the WT $\alpha$-ENaC when co-expressed in oocytes. If, by analogy with these observations, and with roles of the N-termini in assembly of other heterooligomeric channels (Hoshi et al., 1990; Zagotta et al., 1990; Li et al., 1992; Verrall and Hall, 1992; Shen et al.,

1993; Shen and Pfaffinger, 1995; Bixby et al., 1999), the N-terminus of $\beta$-ENaC was important for the interaction(s) between different ENaC subunits, then our findings with the channels containing these mutants can be explained by incomplete assembly. This hypothesis seems plausible considering the fact that, with regard to effects of CFTR, heterodimeric combinations of the WT $\alpha$-subunit with N-terminally truncated $\beta$- or $\gamma$-ENaCs (as well as heterotrimeric $\alpha_{WT}\beta_{\Delta N}\gamma_{\Delta N}$-channels) are reminiscent of the homomultimeric WT $\alpha$-channel.

To conclude, we have identified the N-terminus of the $\alpha$-subunit as a major determinant for the kinetic behavior of both homooligomeric and heterooligomeric ENaCs. Our results also suggest that the carboxy-terminal domains of $\beta$- and $\gamma$-ENaC subunits modulate the kinetics of heterooligomeric channels, but the mechanism underlying this modulation does not completely override the gating the $\alpha$-channel, due to the N-terminus of this subunit. CFTR inhibits amiloride-sensitive channels, at least in part, by modulating their gating, extending the time spent by these channels in their closed state, likely by triggering the same, or closely associated, mechanism as that involving the carboxy-terminal domains of the $\beta$- and $\gamma$-ENaC subunits.

The authors gratefully acknowledge Dr. Kevin L. Kirk (UAB) for constructive criticism of the manuscript.

This work was supported by Cystic Fibrosis Foundation Grants Ismail97IO (to I.I.I.) and Stanto97RO (to B.A.S.), and the National Institutes of Health Grants DK37206 (to Dr. Dale J.Benos, UAB), DK 45881, and DK 51067 (to B.A.S.).

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