Intrinsic gating mechanisms of epithelial sodium channels

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LIDDLE’S SYNDROME is a form of hereditary hypertension produced by mutations within the epithelial sodium channel (ENaC) (1, 17). These mutations result in constitutive channel activation. Both an increase in functional channel number and an increase in single-channel open probability (P₀) have been reported (5–9, 14, 15). The initial description of Liddle’s syndrome identified truncation mutations in the COOH-terminal polypeptide chain of the β-ENaC (and subsequently the γ-ENaC) subunit as being causative for constitutive channel activation (3, 5–9, 14, 15). We proposed the hypothesis that the COOH-terminal chains of β and γ could act as intrinsic channel blockers by serving as an inactivation moiety. Our evidence, obtained in both bilayers (7, 8) and heterologous expression systems (9), supports this type of mechanism. We tested the hypothesis that the functional gating particle comprised the COOH-terminal tails of both the β- and γ-ENaC subunits associated as a two-strand, antiparallel β-sheet. Support for this idea was threefold: 1) the inhibitory effects of adding COOH-terminal β- and γ-ENaC 30-amino acid residue peptides together with ENaC comprising wild-type α- and COOH-terminally truncated β- and γ-subunits produced a greater than additive inhibition of the channel; 2) circular dichroism studies showed that the 30-mer β and γ peptides formed a β-sheet; and 3) when the isoleucines and valines within the 30-mer peptides were replaced by the β-sheet, breaking amino acids proline or aspartic acid, the resulting peptides were unable to affect basal-activated ENaC (8). The paradigm that we have developed for ENaC gating is as follows. Because α-ENaC itself forms a functional sodium channel (4), there must be an intrinsic gating mechanism in α-ENaC alone. We hypothesized that calcium is intimately involved in this process and have presented evidence to this effect (2). The overall gating properties of α-ENaC vs. αβγ-ENaC in bilayers do not differ (7). Because the elimination of the cytoplasmic COOH-terminal tails of either or both of the β- and γ-subunits substantially increases single-channel P₀ (5–9), there must be at least two separate gating processes, one inherent to α-ENaC alone and one conferred onto the complex by the β- and γ-subunits.

To further elucidate the mechanism underlying the COOH-terminal β- and γ-ENaC tail block of ENaC, we tested the hypothesis that a highly conserved region following (or at the most distal end of) the second transmembrane domain (M2) in α-ENaC may act as a putative receptor region for the negatively charged COOH-terminal β- and γ-ENaC tails and, thus, facilitate their interaction with the channel. We have identified a sequence of positively charged amino acids between residues 586 and 591 of human α-ENaC (613–624 of the rat α-ENaC ortholog) (10) that is identical in all five mammalian α-ENaC subunits cloned to date (rat, bovine, human, mouse, and guinea pig) and that is arginine rich (RRFRSRYYWSPGR). This region is conserved in δ-ENaC (RRLRRAWFSWPR) (16) but is not present in either the β- or γ-ENaC subunit or in the ENaC-related Aplysia sodium channel that is gated

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by FMRF-amide (phenylalanine-methionine-arginine-phenylalanine) (12). We hypothesized that this concentration of positive charge may form a site for electrostatic interaction of the negatively charged COOH-terminal β and γ tails and, thus, provide an anchoring point for the peptides somewhere near the internal channel mouth. The peptides may bind at a site away from the actual channel mouth but exert an inhibitory effect via steric hindrance or by inducing a conformational change. In the present experiments, we tested this hypothesis by mutating either two or four of the arginine residues in this sequence to amino acids of opposite charge. Our prediction was that the disruption of the charge distribution in this region would abolish the ability of the COOH-terminal β- and γ-ENaC peptides to inhibit the channel.

We expressed the β-ENaC subunit truncated at amino acid R564 (βR564X) and the γ-ENaC truncated at amino acid R574 (γR574X) in combination with the wild-type rat α-ENaC subunit (i.e., αβR564XγR574X) in Xenopus oocytes. Figure 1 shows representative amiloride-sensitive Na⁺ current traces obtained in voltage-clamped oocytes before and after 1 mM peptide mixture injection. In this series of experiments, the currents obtained from oocytes injected with wild-type α-ENaC and truncated β- and γ-ENaC subunits were greater than the corresponding currents recorded from wild-type αβγ-ENaC injected in oocytes obtained from the same frogs (data not shown). The average inward current levels were approximately twofold increased, as reported earlier (9). Injection of a mixture of β plus γ COOH-terminal peptides (SP30γ plus SP30γ, 1:1, 500 μM each) into oocytes expressing the truncated ENaC constructs (i.e., αβR564XγR574X-ENaC) decreased the current by ~50% at negative potentials. In contrast, the currents in oocytes expressing either of the α double mutations (i.e., αR586E, R587E or αR589E, R591E) or the quadruple mutation in the α-subunit (αR586E, R587E, R589E, R591EβR564XγR574X) were not affected by the same concentration of peptide mixture. As controls, the αβR564XγR574X-ENaC associated current was not affected by water injection or by injection of SP30γ plus a 30-amiino acid peptide identical to SP30β, with substitution of three prolines for one valine and two isoleucines (see Fig. 4). Summary data for the effect of the peptide mixture on wild-type α-subunit plus truncated β- and γ-subunits, two double arginine mutations, and the quadruple mutation (all in combination with the truncated β- and γ-subunits) are shown in Fig. 2. Of these constructs, only the channel containing wild-type α-ENaC was inhibited by 1 mM peptide mixture. In contrast, both double mutants (namely, αR586E, R587E and αR589E, R591E) and the quadruple mutant, in which the four positively charged arginines (R residues) were replaced with negatively charged Glu (E residues), were unaffected by injection of SP30β plus SP30γ (Fig. 2).

As further controls for these experiments, we made a series of α mutations in the same position as the arginines, except that instead of changing the sign from positive to negative (glutamic acid), we maintained the same charge distribution by substitution with lysine (K). In an additional group of oocytes, 1 mM peptide added to αR586K, R587K, R589K, R591KβR564XγR574X, αR586K, R587KβR564XγR574X, or αR586K, R587K, R591KβR564XγR574X inhibited macroscopic currents to the same extent as when added to the wild-type α-subunit. These data are supportive of the hypothesis that this arginine-rich region in the most distal portion of the M2 region plays a role in the interaction of the β- and γ-subunit tails, causing inhibition of the channel.

We used single-channel analysis to explain more precisely the results that we obtained with these COOH-terminal peptides in macroscopic current measurements in heterologously expressing oocytes. Figure 3 shows representative single-channel current

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**Fig. 1.** Representative current traces showing the effects of a 30-mer peptide mixture injection into Xenopus oocytes on amiloride-sensitive Na⁺ currents produced by various mutated α-ENaC (epithelial sodium channel) constructs (left). Records were made 24 h after cRNA injection for all groups of oocytes. Test voltages were stepped from a holding potential of 0 mV to -100 through +100 mV in a 10 mV increment, and each step was held for 500 ms. **Right:** effects of peptide mixture (total concentration 1 mM, 500 μM each of SP30β and SP30γ) on currents. Each experiment was repeated at least 3 times.
traces for αβR564XγR574X, the two double α arginine mutants, and the quadruple mutant in combination with the truncated β- and γ-subunits. The unitary conductances of all of these combinations of mutated and truncated ENaCs averaged 7 pS, demonstrating that there was no effect on the single-channel conductances by introduction of these mutations in the α-ENaC subunit. The peptide mixture only inhibited the single-channel activity (NP₀) of the wild-type α construct (Fig. 3, horizontal bar above traces). The peptide mixture was without effect in either of the double Glu (R→K) α mutants or in the quadruple Glu (R→E) α mutant. The value of NP₀ was decreased to 1.27 ± 0.3 by the peptide mixture from 2.67 ± 0.4 in patches isolated from oocytes expressing αβR564XγR574X-ENaC. In contrast, the value of NP₀ recorded from excised patches with the quadruple mutant was unchanged following peptide exposure (2.91 ± 0.4 and 2.89 ± 0.3). In contrast, the values of NP₀ before and after addition of the control peptide mixture were 1.02 ± 0.19 and 1.14 ± 0.22 (n = 3, Fig. 4).

These direct biophysical measurements of single ENaC demonstrate directly that the COOH-terminal regions of the β- and γ-ENaC subunits function as intrinsic gating particles and that the interaction site occurs at an arginine-rich region located at the most distal portion of the M2 segment of the α-ENaC subunit. Thus these terminal cytoplasmic domains of β- and γ-subunits play an important role in ENaC gating.

Fig. 2. Summary of data showing the effect of SP₃₀β plus SP₃₀γ (1 mM) injection on macroscopic amiloride-sensitive Na⁺ conductance in Xenopus oocytes using different ENaC constructs. Data were averaged amiloride-sensitive Na⁺ conductances; Error bars indicate SE. N is the number of eggs recorded. The conductances in the presence of peptides were normalized to that in the absence of peptides (100%). The percentages of conductance in the presence of peptide mixture (from far left to the far right) are 38.7 ± 8, 48.3 ± 16, 98.8 ± 8, 50.3 ± 19, 103.5 ± 7, 47.2 ± 21, and 96.3 ± 10% of the control before peptide injection. *P < 0.05 compared with the control.

Fig. 3. Representative current traces recorded from inside-out patches of oocytes expressing various mutated α-ENaC constructs. Horizontal bars above the current traces indicate perfusion of the bath with 1 mM SP₃₀β + SP₃₀γ peptide mixture. The pipette medium consisted of 100 mM lithium chloride, and 100 mM lithium chloride was also included in the bath solution. The pH of the medium was adjusted to 7.4 with HEPES buffer at 23°C. The holding potential was −60 mV for each trace.
EXPERIMENTAL PROCEDURES

Construction of α-ENaC mutations. Full-length human αβγ-ENaC cDNA was a gift from Dr. Michael J. Welsh (University of Iowa) (13), and truncated β- and γ-ENaC cDNAs were a gift from Dr. Bernard Rossier (Université et Lausanne, Switzerland) (14). Point mutations in the α-subunit were constructed by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Each set of primers contained the appropriate base changes required to code for either two or four glutamates instead of the wild-type arginine residues. Plasmid cDNA, PCR, in vitro transcription, and bacterial transformation were all done as described previously (11). cDNA products containing the specific mutations were confirmed by dideoxy sequence analysis as well.

Oocyte preparation and electrophysiological recording. Oocytes were removed from anesthetized adult female Xenopus laevis (Xenopus Express, Beverly Hills, FL) by standard technique (9). Follicle cells were removed in OR-2 calcium-free medium (in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl2, 1.0 Na2HPO4, and HEPES 5.0, pH 7.5), with the addition of collagenase. Defolliculated oocytes were washed in both OR-2 (calcium-free) and OR-2 (complete) medium (in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl2, 1.0 CaCl2, 1.0 Na2HPO4, and HEPES 5.0, pH 7.4) and allowed to recover overnight in half-strength Liebovitz’s medium at 18°C. Stage VI oocytes were injected with 50 nl (8.3 ng of the appropriate α-, β-, and γ-ENaC cRNA construct; all subunit mixtures were 1:1:1). Two-electrode voltage clamp and/or single-channel measurements were made 24–48 h postinjection as described previously (9). Oocytes were clamped at a holding potential of 0 mV. The current-voltage relationships were acquired by stepping the holding potential in 10-mV increments from −100 to +100 mV. Current-voltage data were recorded after the monitoring currents were stable, before and after the application of 10 μM amiloride to the bath. Data were sampled at a rate of 1 kHz and filtered at 500 kHz. Data analysis was also as described previously (9). Analysis of single-channel data was performed by using FETCHAN and pSTAT programs of pCLAMP version 8.0 software (Axon Instruments, Burlingame, CA) as previously described (9).

Peptide synthesis and purification. Peptides were synthesized by ResGen (Huntsville, AL). After synthesis, the peptides were subjected to reversed-phase, high-performance liquid chromatography to increase their purity to >90%. Only a single peak was observed on the final chromatograph. The peptides were analyzed for amino acid composition by mass spectroscopy. The sequences for the COOH-terminal 30-amino acid-long β-ENaC (SP30β) and γ-ENaC (SP30γ) peptides were as follows: SP30β, PIPGTPPPNYNTLRLERAFSNQLTDQMLDEL; and SP30γ, PGTPKYNTLRLEAFSNQLTDQMLDEL.

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