Molecular Cloning and Functional Expression of a Novel Amiloride-sensitive Na\textsuperscript{+} Channel\textsuperscript{*}

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We have isolated a cDNA for a novel human amiloride-sensitive Na\textsuperscript{+} channel isoform (called \(\delta\)) which is expressed mainly in brain, pancreas, testis, and ovary. When expressed in Xenopus oocytes, it generates an amiloride-sensitive Na\textsuperscript{+} channel with biophysical and pharmacological properties distinct from those of the epithelial Na\textsuperscript{+} channel, a multimeric assembly of \(\alpha\), \(\beta\), and \(\gamma\) subunits. The Na\textsuperscript{+} current produced by the new \(\delta\) isoform is increased by two orders of magnitude after coexpression of the \(\beta\) and \(\gamma\) subunit of the epithelial Na\textsuperscript{+} channel showing that \(\delta\) can associate with other subunits and is part of a novel multisubunit ion channel.

Amiloride-sensitive sodium channels (ASCs)\textsuperscript{1} are Na\textsuperscript{+}-permeable non-voltage-sensitive ion channels inhibited by the diuretic amiloride. They are abundant and well characterized in epithelial tissues such as kidney, colon, and lung (for review, see Ref. 1) where they control the rate and extent of Na\textsuperscript{+} reabsorption under the regulation of steroid hormones (2–5). The same ASCs also seem to play an important role in taste perception (6). Different mammalian forms of ASCs with different biophysical (conductances, selectivity) and pharmacological properties (sensitivity to amiloride and derivatives) (for review, see Ref. 7) have been characterized recently in thyroid (8), smooth muscle (9), and vascular endothelial cells from brain (10). In addition, amiloride-blockable nonselective ion channels are also important for mechanotransduction (11). Molecular cloning of the highly Na\textsuperscript{+}-selective epithelial Na\textsuperscript{+} channel has demonstrated recently that it is made of at least three homologous subunits called \(\alpha\), \(\beta\), and \(\gamma\) (3, 12–15). Each of these subunits has homologies with the degenerins of the nematode Caenorhabditis elegans (16–19), which after certain mutations cause neurodegeneration. These degenerins are thought to be ion channels (17, 18, 22).

It seems likely that the epithelial amiloride-sensitive Na\textsuperscript{+} channel is the first cloned member of a new family of ion channels which probably includes mammalian homologues of the C. elegans degenerins which might be involved in certain forms of neurodegeneration. This paper reports the molecular cloning and functional expression of a novel human isoform of an amiloride-sensitive Na\textsuperscript{+} channel expressed in brain, testis, ovary, and pancreas.

**MATERIALS AND METHODS**

PCR Amplifications Library Construction and Screening—cDNA was synthesized from human kidney poly(A\textsuperscript{+}) RNA (Clontech) using an oligo(dt) primer with a XhoI restriction site following the protocol supplied with the superscript reverse transcriptase (Life Technologies, Inc.), and a human kidney cDNA library in λZAP (Stratagene) was prepared following standard procedures. A fragment corresponding to nucleotides 42–265 of the expressed sequence tag (GenBank\textsuperscript{TM} accession number T19320) was amplified by PCR, subcloned into Bluescript SK\textsuperscript{(–)} (Stratagene), sequenced, and used to screen the library using Erase-a-Base System (Promega) and sequenced on an Applied Biosystems automatic sequencer. The open reading frame and flanking sequences were sequenced on both strands.

Construction of an Oocyte Expression Vector—An oocyte expression vector (pBSK-SP6-globin) was constructed as follows. The noncoding sequences from Xenopus globin precloned by an SP6 promoter and flanking an EcoRI and XhoI site were amplified from the pEXO vector (3) by PCR using ATTATGGTGACATACATAGCTCAGA and a M13 reverse primer. The PCR product was digested with PstI and ligated into EcoRI/PstI-cut Bluescript SK\textsuperscript{(–)} (Stratagene). The resulting vector was double-digested with ApaI and HindIII and blunt end-re-ligated to remove the XhoI site in the Bluescript vector. In order to remove the long (1.2-kb) 5’-noncoding sequences of \(\alpha\)NaCh containing various start and stop codons, the cDNA was amplified using the Pwo polymerase (Boehringer) with a primer (CAGAATTCCTGCCCGCAATGGC) positioned on the first ATG codon (underlined) of the open reading frame and a primer complementary to the T7 promoter. The PCR product was digested with EcoRI and XhoI and ligated into EcoRI/XhoI-digested pBSK-SP6-globin vector.

Expression in Xenopus Oocytes—RNA was prepared using SP6 or T7 RNA polymerase and the NotI-digested vector as template, and oocytes were injected with 5 to 15 pg of \(\alpha\)NaCh or \(\beta\)NaCh alone or together with 5–15 pg of one or several of the other subunits essentially as described (23). Whole cell recordings were carried out essentially as described (23). Cell-attached recordings were performed on oocytes clamped to 0 mV in high K\textsuperscript{+} medium. Pipettes contained (in mM): NaCl (or LiCl), 140; MgCl\textsubscript{2}, 1; CaCl\textsubscript{2}, 1; Hepes, 10, pH 7.4. Data were sampled at 1 kHz and filtered at 300 Hz for analysis (Biopatch software, Biologic). Single-channel conductances were calculated from i–v relations from 0 mV to –100 mV.

Northern Blots—Human multi-tissue Northern blots containing about 2 \(\mu\)g of poly(A\textsuperscript{+}) RNA normalized for identical \(\beta\)-actin expression in each lane were purchased from Clontech and hybridized with a random prime-labeled Ksp1 fragment (bases 238–737) located just after the first transmembrane region. The blots were hybridized overnight at 65°C in 5 \(\times\) SSC, 10 \(\times\) Denhardt's solution, 0.1% SDS, 100 \(\mu\)g/ml herring sperm DNA, washed with 0.1 \(\times\) SSC, 0.1% SDS at 70°C, and subsequently exposed to Kodak X-OMAT AR film for 14 days at –70°C. The apparent molecular weight of the \(\alpha\)NaCh RNA was calculated using the mobilities of a 0.24–9.5-kb RNA ladder (Life Technologies, Inc.).

All nucleic acid positions in the text refer to positions relative to the A in the ATG initiation codon of the nucleic acid sequence submitted to GenBank\textsuperscript{TM} (accession number U38254).

All comparisons of sequences with data bases were done using the Blast network server at the NCBI (National Center for Biotechnology Information).

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Cloning of a Novel Amiloride-sensitive Na\(^+\) Channel

**Fig. 1.** Protein sequence of α\(_{\text{NaCh}}\) and comparison with β\(_{\text{NaCh}}\), γ\(_{\text{NaCh}}\), and γ\(_{\text{NaCh}}\). a, alignment of α\(_{\text{NaCh}}\) with human α\(_{\text{NaCh}}\), β\(_{\text{NaCh}}\), and γ\(_{\text{NaCh}}\). Residues identical or similar to the corresponding amino acid in the α subunit are printed white on black background, respectively. The putative transmembrane regions for α\(_{\text{NaCh}}\) are labeled with bars. For MII, the hydrophobic region is longer than the same 20 amino acids required for an α helix to span the membrane. The sequence which was shown to participate in the formation of the ionic pore of α\(_{\text{NaCh}}\) (22) is marked by a black bar, and flanking hydrophobic regions by gray bars. The sequence for α\(_{\text{NaCh}}\) is from GenBank™ (accession number X76180), and those for β\(_{\text{NaCh}}\) and γ\(_{\text{NaCh}}\) are from EMBL (accession numbers X877159 and X87160). The sequences were aligned using the GCG Pileup program, and identities shown in a using the Distances program (GCG) with Kimura substitution numbers X877159 and X87160. The sequences were aligned using the GCG Pileup program, and identities were calculated with the GCG Distances program without correction for multiple substitutions. The sequences for the degenerins deg1 and mec10 used are GenBank™ accession numbers L34414 and L25312, respectively.

**RESULTS AND DISCUSSION**

In order to identify novel homologues of the epithelial Na\(^+\) channel (NaCh), the sequences of the cloned subunits (α\(_{\text{NaCh}}\), β\(_{\text{NaCh}}\), γ\(_{\text{NaCh}}\)) have been compared with the data base of expressed sequence tags. We found one good matching partial cDNA sequence (GenBank™ accession number T19320) in this data base. A fragment of this sequence was amplified by PCR from human kidney cDNA and used to screen a human kidney cDNA library. A positive clone of 3.4 kb was isolated and sequenced. It contains an open reading frame of 1914 bases corresponding amino acid in the α subunit than to β\(_{\text{NaCh}}\) and γ\(_{\text{NaCh}}\). The sequences were aligned using the GCG Pileup program, and identities shown in a using the Distances program (GCG) with Kimura substitution numbers X877159 and X87160. The sequences were aligned using the GCG Pileup program, and identities were calculated with the GCG Distances program without correction for multiple substitutions. The sequences for the degenerins deg1 and mec10 used are GenBank™ accession numbers L34414 and L25312, respectively.

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**The homology with the α, β, and γ subunit (27–37% identity; Fig. 1c) is rather low and lies in the same range as observed between α\(_{\text{NaCh}}\), β\(_{\text{NaCh}}\), and γ\(_{\text{NaCh}}\) (29–36% identity; Fig. 1c). Nevertheless, the homology and phylogenetic analysis (Fig. 1b) places this new isoform, named α\(_{\text{NaCh}}\), closer to the α subunit than to β\(_{\text{NaCh}}\) and γ\(_{\text{NaCh}}\). The α\(_{\text{NaCh}}\) is, as α\(_{\text{NaCh}}\), β\(_{\text{NaCh}}\), and γ\(_{\text{NaCh}}\), about 20% identical with the degenerins mec10 and deg1 of C. elegans (Fig. 1c).

α\(_{\text{NaCh}}\) has a hydrophobicity profile similar to α\(_{\text{NaCh}}\), β\(_{\text{NaCh}}\), and γ\(_{\text{NaCh}}\) and to the degenerins with two hydrophobic regions (M1 and MII, Fig. 1a) long enough to span the plasma membrane. Together with the sequence homologies, this suggests a transmembrane topology identical with that proposed for α\(_{\text{NaCh}}\) (20) with intracellular amino and carboxyl termini and a large cysteine-rich extracellular loop between M1 and MII.

When expressed alone in Xenopus oocytes, α\(_{\text{NaCh}}\) induced a small (38 ± 5 nA, n = 16) but very reproducible amiloride-sensitive Na\(^+\) current (Fig. 2) with macroscopic properties (pharmacology, selectivity) clearly distinct from those of α\(_{\text{NaCh}}\) when expressed in the same conditions (13).

The first difference concerns the pharmacology. K\(_{\text{b}}\) values (Fig. 2c) for the diuretic amiloride (2.6 μM) and for benzamil (0.27 μM) were about 30 times higher than those for α\(_{\text{NaCh}}\) (K\(_{\text{b}}\); amiloride = 80 nM; K\(_{\text{b}}\); benzamil = 7 nM). The second difference was the ionic selectivity. The α\(_{\text{NaCh}}\) channel was more permeable for Na\(^+\) than for Li\(^+\) (I\(_{\text{Na}}\)/I\(_{\text{Li}}\) = 0.6) unlike the human α\(_{\text{NaCh}}\) or rat α\(_{\text{NaCh}}\) which have a higher permeability for Li\(^+\) than for Na\(^+\) (I\(_{\text{Li}}\)/I\(_{\text{Na}}\) = 2) (Fig. 2b). α\(_{\text{NaCh}}\) was insensitive to ethylisopropylamiloride (Fig. 3b), a potent inhibitor of the Na\(^+\)/H\(^+\) exchanger (21), at concentrations below 10 μM.
Like αNaCh, δNaCh is virtually impermeable for K\(^+\). No amiloride-sensitive current could be detected when Na\(^+\) was substituted by K\(^+\) (Fig. 2b), and the i − V curve shows a positive reversal potential (+49 ± 7 mV, n = 5) (Fig. 2d).

Since the epithelial Na\(^+\) channel is known to be a multisubunit assembly and since αNaCh alone also induces only small currents when expressed in Xenopus oocytes without βNaCh and γNaCh (3, 12–15), we examined whether any of the other known human subunits (α, β, or γ) increases the δNaCh current (Fig. 3a). Unlike αNaCh for which coexpression of just the γ subunit increases the current by one order of magnitude (3, 14), none of the α, β, or γ subunits alone altered or increased the δNaCh current when coexpressed with δNaCh. However, coexpression of both the β and γ subunits with the δNaCh increased the Na\(^+\) current by 50-fold (1.94 ± 0.4 μA, n = 7), an amplification that lies in the same range as that reported after coexpression of αNaCh with βNaCh and γNaCh (14).

The macroscopic properties (pharmacology, ionic selectivity) of δβγNaCh were indistinguishable from those of δNaCh. Together with the fact that the macroscopic properties of αNaCh are also not altered by coexpression of βNaCh and γNaCh (3, 14), this suggests either that αNaCh and δNaCh are the pore-forming subunits or that low amounts of endogenous βNaCh and γNaCh-like subunits are present in the oocyte and are responsible for the small currents observed after expression of αNaCh or δNaCh alone.

The single-channel conductance (Fig. 3) for Na\(^+\) of δβγNaCh was 11.6 ± 0.4 pS (n = 8). It was clearly different from that of αβγNaCh (4.8 ± 0.3 pS, n = 6). The δβγNaCh conductance for Li\(^+\) (6.8 ± 0.5 pS, n = 4) was nearly identical with that of αβγNaCh (7.3 ± 0.2 pS, n = 4). The δβγNaCh channel, like αβγNaCh, was highly selective for Na\(^+\) versus K\(^+\) (pNa\(^+/\)pK\(^+\) > 50). The gating of δβγNaCh was slow (τ\(_{\text{open}}\) = 3.3 ± 1.5 s, τ\(_{\text{close}}\) = 1.9 ± 0.7 s, n = 5; Fig. 3b), and the open probability did not show a marked voltage dependence (P\(_o\) = 0.46 ± 0.05 at −20 mV and 0.49 ± 0.02 at −100 mV, n = 3).

It is particularly interesting that, despite their low homology (37% identity), both αNaCh and δNaCh can associate with βNaCh and γNaCh to form a functional channel. Sequence comparisons between αNaCh and δNaCh (Fig. 1) reveal some motifs that are well conserved and which are not found in βNaCh and γNaCh. Those “common” α and δ sequences, and particularly the sequence just before MI (Fig. 1a), are probably important elements for the functional association of αNaCh or δNaCh with βNaCh and γNaCh.

The tissue distribution of δNaCh mRNA was analyzed by Northern blot (Fig. 4). The highest expression levels of the 5.5-kDa mRNA were found in testis, ovary, pancreas, and brain. Those are tissues in which to our knowledge no amiloride-sensitive Na\(^+\) channels have been described yet. Small amounts of δNaCh-mRNA can be detected in all other tissues examined except in spleen and small intestine. The dominant tissue distribution of δNaCh is clearly epithelial, and, in kidney (the tissue we cloned δNaCh from), there are only small amounts of δNaCh-mRNA present. Therefore, it does not seem likely that the principal role of this new channel is to be searched in epithelia.

The pharmacological and biophysical properties of δNaCh do not really match those of any of the amiloride-sensitive Na\(^+\) channels described so far. Like αNaCh, the major subunit of the epithelial Na\(^+\) channel, δNaCh, can associate with βNaCh and γNaCh to form a multisubunit ion channel. Whether this δβγ subunit combination is the one actually present in vivo or whether yet unknown subunits form a channel together with δNaCh requires further investigation.

The presence of δNaCh in brain is particularly interesting, because the C. elegans degenerins (16–19) which are homologues to NaCh are expressed in neurons. A more detailed localization of δNaCh especially in brain might clarify the physiological role of this new amiloride-sensitive Na\(^+\) channel.
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