Cloning and Expression of a Novel Human Brain Na\(^+\) Channel*

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We have cloned a novel cDNA from human brain which encodes a non-voltage-dependent Na\(^+\) channel (BNC1). BNC1 has some sequence similarity (24–28%) with a new channel family that includes subunits of the mammalian epithelial Na\(^+\) channel, the Caenorhabditis elegans degenerins, and the Helix aspersa FMRF-amide-gated Na\(^+\) channel. Like other family members it is inhibited by amiloride. However, its predicted structure differs from other family members, its discrimination between Na\(^+\) and Li\(^+\) is different, and in contrast to other mammalian family members, coexpression with other cloned subunits of the family does not increase current. BNC1 has a unique pattern of expression with transcripts detected only in adult human brain and in spinal cord. Thus, BNC1 is the first cloned member of a new subfamily of mammalian Na\(^+\) channels. The function of BNC1 as a non-voltage-gated Na\(^+\) channel in human brain suggests it may play a novel role in neurotransmission.

Recent studies have identified a new family of Na\(^+\) channels whose characteristic features include Na\(^+\) selectivity, inhibition by amiloride, and a conserved primary structure (1–11). Family members contain 500 to 800 residues. Sequence analysis and studies of topology suggest that the amino and carboxyl termini are intracellular, that there are two hydrophobic regions that traverse the membrane (M1 and M2), and that between M1 and M2 there lies a large cytoplasmic extracellular domain (12–14). The best characterized members of this family are the amiloride-sensitive epithelial Na\(^+\) channels (ENaC) that control Na\(^+\) and fluid absorption in the kidney, colon, and lung. ENaC channels are constructed from at least three homologous subunits (\(\alpha, \beta, \gamma\), and \(\gamma\)ENaC) (4–9). These channels may also be involved in detection of salty taste (15). A closely related subunit, \(\delta\)NaCh, is expressed in pancreas, testis, ovary, and brain. \(\delta\)NaCh generates Na\(^+\) channels when coexpressed with \(\beta\) and \(\gamma\)ENaC (10), suggesting that it may be part of the ENaC subfamily of channels. Several family members have been discovered in C. elegans, including MEC-4, MEC-10, and DEG-1, which when mutated produce a touch-insensitive phenotype (1–3). Although the function of these gene products has not been established, several observations suggest that they form ion channels: their sequences are similar to the ENaC subunits; genetic evidence suggests that three gene products are required for function (3); M2 of \(\delta\)ENaC can substitute for M2 of MEC-4 (16); and specific mutations cause ballooning cellular degeneration (1, 2) similar to that found with overexpression of active ENaC subunits (17). Based on this ability to produce cell degeneration, family members in C. elegans are called “degenerins.” The most recent addition to this family is a Phe-Met-Arg-Phe-NH\(_2\) (FMRF-amide)-stimulated Na\(^+\) channel (FaNaCh) cloned from Ha\(_\text{dix}\) (11).

Here we report the cloning and expression of a novel member of the family which is expressed in human brain. We name it BNC1 for Brain Na\(^+\) Channel, and 1 with the expectation that additional subunits will be discovered in the future.

EXPERIMENTAL PROCEDURES

Cloning—A complete BNC1 cDNA was obtained by extending an expressed sequence tag (GenBank\(^\text{TM}\) accession number Z45660) in the 5' direction using rapid amplification of cDNA ends (RACE) technique according to the protocol provided with the Marathon cDNA Amplification Kit from Clontech. Human brain cDNA which had been tagged with an adapter primer at the 5' end (Clontech) was used as template in 5' RACE reactions. In brief, the tagged cDNA was used in a PCR reaction with a sense primer corresponding to the tag sequence and a gene-specific antisense primer corresponding to nucleotides 256–282 of the EST sequence. The 3' end of the gene-specific primer spanned the 3' end of the cDNA and contained a sequence complementary to the stop codon. RACE PCR reactions were done using reagents in the Advantage cDNA PCR core kit (Clontech) which contains a combination of Klen-Taq-1 and Deep Vent DNA polymerases and TaqStart antibody. Thermal cycling was done in a Perkin Elmer DNA Thermal Cycler using a program of one cycle at 94°C for 1 min; 5 cycles of 94°C for 30 s and 72°C for 4 min; 5 cycles of 94°C for 30 s and 70°C for 4 min; then 20–25 cycles of 94°C for 20 s and 68°C for 4 min. PCR products were purified on an agarose gel using \(\beta\)-agarase from New England Biolabs, cloned into the pCR\(^\text{II}\) vector (Invitrogen), and sequenced. DNA sequencing was done on an Applied Biosystems automated Sequencer using fluorescent dye-labeled terminators. An 1809-bp fragment was obtained from the 5' RACE reaction which contained 270 nucleotides of upstream untranslated sequence and a 1539-bp open reading frame extending to the 3' stop codon. This fragment was digested in its entirety out of the pCR\(^\text{II}\) vector as a NotI/KpnI fragment and ligated into the compatible sites of the PMT3 vector for expression in oocytes (18). Oligonucleotides were prepared on an automated Applied Biosystems 3800A synthesizer. Relationship of proteins in the phylogenetic tree was derived using the Pileup alignment program from Genetics Computer Group (GCC). The diagram was generated using the Distances program (GCC) with Kimura substitution, followed by the Growtree program with the UPGMA option.

Northern Blot Analysis—Northern blots contained 2 \(\mu\)g of poly(A\(^+\)) RNA isolated from specific adult human tissues or from sections of the...
Brain (Clontech). Probes were prepared by random primer labeling (Pharmacia Biotech Inc.). PCR primers specific for the 5' and 3' ends of the protein coding sequence of the BNC1 cDNA were used in a PCR reaction to generate a fragment containing the entire coding sequence of BNC1. This fragment was cloned into the pCR® vector and used to probe the multiple tissue blots. An EcoRI/SphI 460-bp fragment was isolated from the 5' end of the coding region of BNC1. A 299-bp PCR product corresponding to the 3' end of the coding region of BNC1 was cloned for use as a 3' end specific probe. Filters were hybridized overnight at 42°C in a buffer containing 50% formamide, 2× SSPE, 2% SDS, 10× Denhardt's solution, and 100 μg/ml salmon sperm DNA. Filters were washed with 0.1× SSC, 0.1% SDS at 55°C and exposed to X-ray film for 4 days at −80°C.

Expression of BNC1 in Xenopus laevis Oocytes—BNC1 was expressed in Xenopus oocytes by nuclear injection of BNC1 cDNA cloned into pMT3 (0.2–0.3 ng). Control oocytes were injected with H2O, oocytes were bathed in 116 mM NaCl, 2 mM KCl, 0.4 mM CaCl2, 5 mM MgCl2, 5 mM Hepes (pH 7.4 with NaOH). To determine ionic selectivity, NaCl was replaced with LiCl or KCl. Current-voltage relationships were determined by stepping from a holding potential of −60 mV to potentials between −100 and +40 mV for 1 s. Amiloride-sensitive current was obtained by subtracting current during exposure to a maximal concentration of amiloride (100 μM) from current prior to amiloride addition. Phe-Met-Arg-Phe-NH2 (FMRF-amide), Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH2 (F-8-F-amide), and Ala-Gly-Glu-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH2 (A-18-F-amide) were added to the bathing solution at 1–30 μM for 1 s.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis—To identify new mammalian Na+ channels, we used the BLAST sequence alignment program (19) to search the data base of expressed sequence tags (EST) with the amino acid sequences of hENaC and the degenerins. We found a 299-nucleotide sequence (GenBank™ accession number U50352) that codes for a protein with similarities of 24.2–26.6% identical with FaNaCh, 24.4–25.4% identical with FaNaCh, and 24.2–26.6% identical with FaNaCh and 24.4–25.4% identical with FaNaCh. Although these sequences of FaNaCh have been detected in brain, they are much more prevalent in other tissues. α- and γENaC are most abundant in epithelia of kidney, colon, and lung (4–9), and δENaC is most abundant in testis, ovary, and pancreas (10). Expression of nonmammalian members of the family has been reported in excitable tissue. Transcription of FaNaCh occurs in muscle and nervous tissue of C. elegans (11), and the degenerins are expressed in the peripheral and central nervous system of C. elegans (1–3).

When the entire coding region of BNC1 was used as a probe, we detected two transcripts, 2.7 and 3.7 kb in length (Fig. 2, A and B). In general, relative hybridization to the two transcripts was similar in most brain regions, although there was a greater relative abundance of the larger transcript in the cerebellum, medulla, spinal cord, corpus collosum, hypothalamus, substan-
HypothesisthatBNC1isaNa¹ channel.

...exposure of were exposed to film for 4 days; a 7-day exposure of BNC1 is a Na⁺ channel.

...coding sequence of BNC1 as described under "Experimental Procedures." Blots were exposed to film for 4 days; a 7-day exposure of B showed that both transcripts were evident to some extent in every lane.

...tia nigra, and thalamus. To investigate the relationship between the two transcripts, we prepared probes from the 5' and 3' regions of BNC1 cDNA (corresponding to the amino and carboxyl termini of the predicted protein) and hybridized them to a Northern blot containing human brain poly(A)⁺ RNA (Fig. 3). Whereas the 3' probe hybridized with both transcripts, the 5' probe hybridized with the 2.7-kb transcript only. These data indicate that the cDNA reported here is produced by the smaller transcript. There are at least two possible explanations for the presence of two transcripts. First, alternative splicing at the amino terminus might generate two transcripts from a single gene. Second, there may be two genes with very similar sequences corresponding to the 3' end of BNC1. Further investigation is necessary to distinguish between these alternatives. In either case, the data suggest the possibility of structural and thus functional complexity with multimeric channel proteins.

Expression of BNC1 in Xenopus Oocytes—Because of its homology with ENaC and FaNaCh Na⁺ channels, we tested the hypothesis that BNC1 is a Na⁺ channel. Expression of BNC1 in Xenopus oocytes generated a small inward current (holding potential = −60 mV) that was reversibly inhibited by amiloride (14.0 ± 2.7 nA, n = 12, Fig. 4A). There was no amiloride-sensitive current in control (H₂O-injected) oocytes (Fig. 4B). The BNC1 current was highly selective for Na⁺ relative to K⁺; the reversal potential was 35 ± 6 mV (n = 6) in NaCl bathing solution, and the amiloride-sensitive current was abolished by replacing Na⁺ with K⁺ in the bathing solution (Fig. 4B). The Na⁺ current was inhibited by amiloride with half-maximal inhibition at 147 ± 23 nM (Fig. 4C).

When we replaced Na⁺ with Li⁺, we measured equal currents through BNC1 channels (Fig. 4D). This differs from αβγENaC and αENaC which are 2-fold more conductive to Li⁺ than to Na⁺ (Fig. 4D), and from FaNaCh and δNaCh which are more conductive to Na⁺ than to Li⁺ (9–11). It was previously shown that Ser⁵⁸⁹ in αENaC was important for Na⁺/Li⁺ selectivity; mutation to Ile increased Na⁺ conductance relative to Li⁺ (21). The analogous residue in BNC1 (and δNaCh) is alanine (Ala⁴⁴²), suggesting that this residue might help determine relative Na⁺/Li⁺ conductivity in BNC1 as well as in other family members.

**CONCLUSION**

BNC1 is a novel member of the ENaC/degenerin family. However, it has several significant differences from other...
two-electrode voltage clamp 1 day after injection at a holding potential injected with cDNA encoding BNC1, and current was measured by structure; it does not discriminate between Na+ cloned members of the family: it has a different predicted Oocytes expressed BNC1 (magnitudewas small. There are at least two explanations. It is that BNC1 may be the first cloned member of a new coexpressed with subunits of ENaC. These considerations sug-

Fig. 4. Expression of BNC1 in Xenopus oocytes. Oocytes were injected with cDNA encoding BNC1, and current was measured by two-electrode voltage clamp 1 day after injection at a holding potential of −60 mV. A, representative current trace. Amiloride (100 μM) was present during time indicated by bar. B, current-voltage relationships for amiloride-sensitive current from representative oocytes expressing BNC1 or injected with H2O (Control). Oocytes were bathed in Na+, Li+, or K+-containing solution, as indicated. Data are plotted relative to current in NaCl. Oocytes expressed BNC1 (n = 9) or αβγhENaC (“hENaC,” n = 4), as indicated. E, amiloride-sensitive current in oocyte expressing ENaC subunits with or without BNC1, as indicated. n = 5–16 for each except αβγhENaC where n = 3.

cloned members of the family: it has a different predicted structure; it does not discriminate between Na+ and Li+ as current carriers; expression was detected only in the central nervous system; and BNC1 current is not augmented when it is coexpressed with subunits of ENaC. These considerations suggest that BNC1 may be the first cloned member of a new subfamily of mammalian Na+ channels.

Although expression of BNC1 generated a Na+ current, the magnitude was small. There are at least two explanations. It is possible that other, as yet unidentified, subunits might be required to produce a fully functional channel complex. The situation may be analogous to eENaC which generates small currents when expressed alone, but produces large currents when coexpressed with β and γENaC (5, 7). It is also possible that BNC1 might require activation by an agonist or neurotransmitter, just as FaNaCh is stimulated by FMRF-amide (11). This latter possibility seems particularly attractive because of its expression in brain. Moreover, ligand-regulated activity rather than constitutive activity would be more consistent with neuronal expression, because constitutive non-voltage-dependent Na+ channel activity could depolarize the cell, thereby disrupting signal transduction, or it could cause cell toxicity. It is interesting to speculate that the large cysteine-rich extracellular domain of BNC1 might have a receptor function. Certainly the large size and presence of multiple cysteine residues is reminiscent of other receptor proteins. Identification of other brain Na+ channel subunits and/or receptor ligands should help us better understand the function of BNC1 and the role of non-voltage-gated Na+ channels in the central nervous system.

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