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9. Bone marrow from 11- to 13-week-old BALB/c mice, treated with 5-FU (150 mg per kilogram of body weight) 6 days before harvest, was cultured at 1 × 10^6 cells per milliliter in Iscove's medium, 10% FCS, 50 μM 2-mercaptoethanol (2-ME), murine IL-3 (0.5 ng/ml), and human IL-6 (50 ng/ml). After 24 hours, media were removed, and cells were resuspended in helper-free retroviral stocks prepared in the same media. Retroviral stocks were prepared as described [A. J. Muller *et al.*, *Mol. Cell. Biol*, **11**, 1785 (1991)] and were supplemented with polybrene (4 μg/ml) and maintained in the same concentrations of IL-3 and IL-6. Fresh media that contained retroviruses were added every 6 hours for up to 48 hours. After a total of 72 hours in liquid culture, cells were washed three times and seeded at 1 × 10^5 cells per milliliter in soft agar.
10. Agar assay was performed as described [T. Lugo and O. N. Witte, *Mol. Cell. Biol.* **9**, 1263 (1989)] with the following modifications: After infection, cells were washed and resuspended at 1 × 10^5 cells per milliliter, and 2 ml of cell suspension was seeded per well in soft agar supplemented with either IL-3 or SLF. The threshold concentration of growth factor necessary to stimulate agar colony formation varied between individual experiments. In each experiment, we determined the optimal concentration of growth factor necessary to preferentially support the growth of *bcr-abl*–expressing cells by supplementing cultures with serial dilutions of either murine IL-3 (1 to 500 pg/ml) or rat SLF (0.3 to 100 ng/ml). Triplicate cultures were established at each concentration of growth factor. Each experiment also included cultures that contained no exogenously added cytokines.
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14. Despite repeated attempts, we were unable to derive erythroid outgrowths from cells in the mixed-phenotype colonies; the *bcr-abl* oncogene may have had a limited effect on the growth of cells in the erythroid lineage, which is correlated with no increase in the erythroid pool during the chronic phase of CML (*1*).
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16. The MG 35.3 cell line was established by infecting bone marrow, which was taken from mice pretreated with 5-FU (150 mg per kilogram of body weight) 6 days before harvest, with helper-free retroviral stocks that expressed $p210^{bcr\text{-}abl}$. After infection, cells were seeded onto S-17 bone marrow stromal cell layers (*21*). After 4 weeks in culture, cells were subcloned by limiting dilution, in the absence of stromal cell layers, in RPMI 1640 media that contained only 5% FCS and 50 μM 2-ME. The MG 35.3 subclone was maintained in this media for over a year before inoculation into mice
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24. We thank J. Braun, D. Saffran, C. Sawyers, and I. Weissman for critical review of the manuscript; N. Parada for photography, I. McNiese and K. Zsebo (Amgen) for murine GM-CSF, rat SLF, human G-CSF, human IL-6, and human erythropoietin. M.L.G. is a fellow of the Leukemia Society of America. O.N.W. is an Investigator of the Howard Hughes Medical Institute. Supported by grants from the National Cancer Institute to O.N.W.

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Primary Structure and Functional Expression of the β_1 Subunit of the Rat Brain Sodium Channel

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Voltage-sensitive sodium channels are responsible for the initiation and propagation of the action potential and therefore are important for neuronal excitability. Complementary DNA clones encoding the β_1 subunit of the rat brain sodium channel were isolated by a combination of polymerase chain reaction and library screening techniques. The deduced primary structure indicates that the β_1 subunit is a 22,851-dalton protein that contains a single putative transmembrane domain and four potential extracellular N-linked glycosylation sites, consistent with biochemical data. Northern blot analysis reveals a 1400-nucleotide messenger RNA in rat brain, heart, skeletal muscle, and spinal cord. Coexpression of β_1 subunits with α subunits increases the size of the peak sodium current, accelerates its inactivation, and shifts the voltage dependence of inactivation to more negative membrane potentials. These results indicate that the β_1 subunit is crucial in the assembly, expression, and functional modulation of the heterotrimeric complex of the rat brain sodium channel.

Voltage-sensitive sodium channels are the membrane proteins responsible for the rapid influx of Na^+ during the rising phase of the action potential in excitable cells (*1*). The major form of the Na^+ channel in rat brain is a heterotrimeric complex of an α subunit (260 kD), a noncovalently associated β_1 subunit (36 kD), and a disulfide-linked β_2 subunit (33 kD) (*2*). Sodium channels in eel electroplax and chicken heart are thought to consist of a single α subunit (*3*), whereas Na^+ channels in rat skeletal muscle and rat heart are heterodimeric, composed of α- and β_1-like subunits (*4*). Four rat brain Na^+ channel α-subunit isoforms have been identified by cloning and sequencing (*5*, *6*). There are also multiple subtypes of β_1 subunits expressed in rat brain, sciatic nerve, skeletal muscle, and heart (*7*). Expression of rat brain Na^+ channel α subunits alone in *Xenopus* oocytes (*6*, *8–10*) or in mammalian cells (*11*) yields functional Na^+ channels that activate and inactivate in response to depolarization. However, brain Na^+ channels expressed in *Xenopus* oocytes from RNA encoding α subunits inactivate slowly compared to Na^+ channels in neurons (*6*, *8*,

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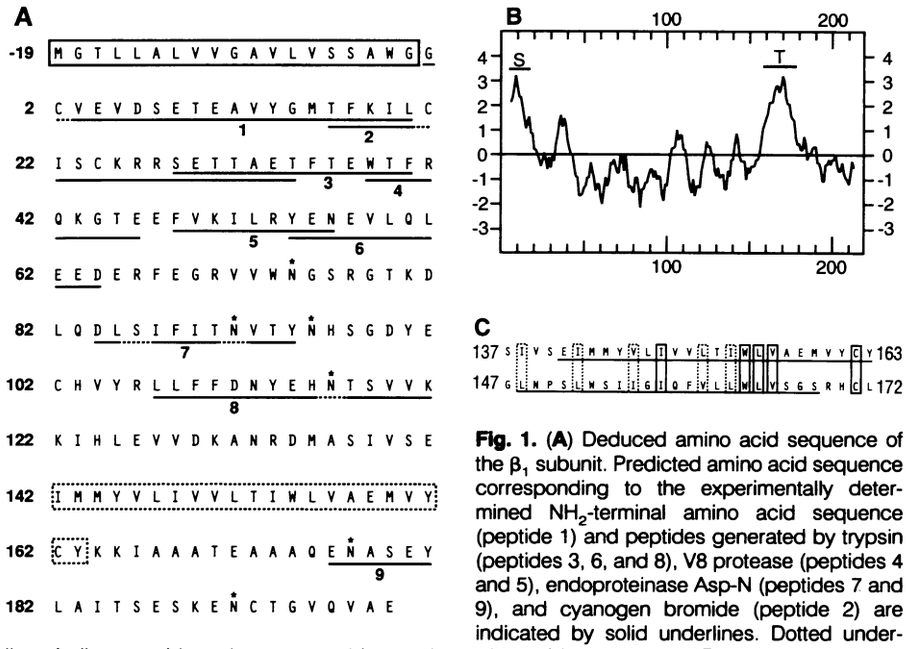

Fig. 1. (**A**) Deduced amino acid sequence of the β_1 subunit. Predicted amino acid sequence corresponding to the experimentally determined NH_2-terminal amino acid sequence (peptide 1) and peptides generated by trypsin (peptides 3, 6, and 8), V8 protease (peptides 4 and 5), endoproteinase Asp-N (peptides 7 and 9), and cyanogen bromide (peptide 2) are indicated by solid underlines. Dotted underlines indicate residues that were ambiguous by amino acid sequencing. (**B**) Hydropathy profile computed according to Kyte and Doolittle (*21*). The window size is six residues plotted at one-residue intervals. The position of the signal peptide is labeled "S." The position of the predicted transmembrane segment is labeled "T." (**C**) Comparison of amino acid sequences of Na^+ channel β_1 and Ca^{2+} channel δ subunits. Putative transmembrane segments are indicated by solid underlines. Identical amino acids are shown inside solid boxes; closely related amino acids are shown inside dashed boxes. Three additional short sequences containing three or four identical or closely related amino acids in the extracellular domains of β_1 and δ were found: 76 to 79 of β_1 (SRGT) compared to 90 to 93 of δ (SKGT); 106 to 108 β_1 (RLL) compared to 98 to 100 of δ (RLL); and 110 to 112 of β_1 (FDN) compared to 129 to 131 of δ (FDN).

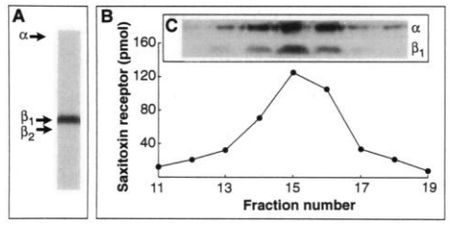

Fig. 2. (**A**) Immunospecific identification of β_1 subunits. Purified Na^+ channels were analyzed by immunoblotting with the antibody to the NH_2-terminal sequence of purified β_1 subunits (*13*). The migration positions of the α, β_1, and β_2 subunits on silver-stained gels are shown by arrows. (**B**) Rat brain Na^+ channels purified by ion exchange, hydroxylapatite, and wheat germ agglutinin affinity chromatography were fractionated by sucrose gradient sedimentation, and aliquots from each fraction were analyzed for [^{3}H]saxitoxin binding by rapid gel filtration (*13*). (**C**) Fractions from the same sucrose gradient sedimentation were analyzed by immunoblotting with antibodies directed against the α and β_1 subunits (*13*).

10). Coinjection of low molecular weight brain mRNA increases the size of the Na^+ current, shifts the voltage dependence of inactivation, and restores normal rapid inactivation (*6*, *12*), suggesting a possible requirement for β_1 or β_2 subunits for efficient and accurate functional expression of brain Na^+ channels. We now describe the primary structure of the β_1 subunit of brain Na^+ channels and show that it modulates functional expression of α subunits.

For amino acid sequence analysis, Na^+ channels were purified from rat brain, the β_1 subunits were isolated, and the NH_2-terminal amino acid sequence was determined to be G[E/C]VEVDSETEAVYGMTFKIL (*13*, *14*). Peptide fragments of the β_1 subunit were prepared by cyanogen bromide cleavage or by digestion with trypsin, endoproteinase Asp-N, or V8 protease (*13*), and amino acid sequences of eight peptides (Fig. 1, underlined) were determined.

To verify that the NH_2-terminal sequence was that of the β_1 subunit, we synthesized a peptide corresponding to the initial 18 amino acids with glutamate in position 2 and norleucine in position 15, coupled to bovine serum albumin, and used the conjugate for production of a polyclonal antiserum (*15*). Antibodies from this serum recognized a single protein band in purified Na^+ channel preparations in the position of the β_1 subunit (Fig. 2A). This protein comigrated precisely with the α subunit and the saxitoxin binding activity of the Na^+ channel complex in sucrose gradient sedimentation (Fig. 2, B and C).

To isolate cDNA clones encoding the β_1 subunit of the rat brain Na^+ channel, we amplified a 102–base pair (bp) cDNA encoding the NH_2-terminal 34 amino acids of the β_1 subunit from rat brain cDNA by the polymerase chain reaction (PCR) with β_1-specific degenerate oligonucleotides (*16*). This PCR product was then labeled with ^{32}P and used to screen 1×10^6 plaques of a λZap II rat brain cDNA library (*17*). Two independent clones of approximately 1400 bp, pβ1.C1Aa and pβ1.22A, were analyzed to determine the nucleotide sequence encoding the β_1 protein (*18*). The translation start site was assigned on the basis of homology to consensus sequences for translational initiation in higher eukaryotes (*19*). This resulted in a 656-bp open reading frame coding for 218 amino acids including the NH_2-terminal sequence of β_1 as well as the sequences of eight additional peptides of the β_1 subunit as determined by protein sequencing (Fig. 1A). The NH_2-terminal sequence of the purified β_1 subunit is preceded by a segment of 19 amino acid residues with the properties of a leader sequence (Fig. 1A, box). The molecular mass calculated from the deduced amino acid sequence of the mature core protein with the signal sequence removed is 22,851 daltons, which is in close agreement with the observed molecular mass of 23 kD for the deglycosylated β_1 subunit as determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*20*). Kyte-Doolittle hydrophobicity analysis (Fig. 1B) (*21*) of the deduced amino acid sequence predicted a single transmembrane domain spanning residues 142 to 163 (Fig. 1A, dotted box). The presence of an NH_2-terminal signal sequence indicated that the NH_2- and COOH-termini are most likely located on the extracellular and intracellular sides of the membrane, respectively, separated by a single transmembrane domain. Four of the six potential N-linked glycosylation sites (Fig. 1A, asterisks) are located in the extracellular domain, which is in agreement with biochemical results that indicate three or four N-linked carbohydrate chains in the mature β_1 glycoprotein of 36 kD (*20*).

A comparison of the nucleotide and deduced amino acid sequences of the β_1 subunit with sequences in the Swiss-Protein and GenBank/European Molecular Biology Laboratory (EMBL) databases revealed no significant homologies (*22*), indicating that the β_1 subunit is not closely related to other ion channel subunits. Of the previously described subunits of voltage-gated ion channels, the β_1 subunit of the Na^+ channel most closely resembles the δ subunit of

the skeletal muscle calcium channel (*23*) in that it has a single membrane-spanning segment, a glycosylated extracellular domain, and a core molecular mass of about 23 kD, although the δ subunit is derived from a 170-kD $\alpha_2\delta$ precursor by posttranslational proteolytic cleavage. A closer comparison of the amino acid sequences of β_1 and δ revealed weak amino acid sequence similarity within and adjacent to the transmembrane segment (Fig. 1C), which may contribute to the similar functional effects of the β_1 and $\alpha_2\delta$ subunits described below. However, in the remainder of the molecule, only three sequences of three to four identical amino acids in the extracellular domain were found (Fig. 1C), indicating at most a distant relation between the two proteins.

To examine the tissue-specific expression of mRNAs encoding the β_1 subunit and related proteins, we isolated total RNA from various rat tissues, eel brain, and eel electroplax, and subjected it to Northern blot analysis (*24*) (Fig. 3). A strongly hybridizing band of 1400 bp was detected in rat brain and rat spinal cord. A similar band of moderate intensity was observed in RNA from rat heart, and weakly hybridizing bands were seen in RNA from rat skeletal muscle and from 3-day-postnatal rat brain after prolonged exposure of the autoradiogram. No specific hybridization was observed in rat liver, eel electroplax, or eel brain, although the background hybridization caused by the approximate comigration of the mRNA for β_1 subunit with 18S RNA and the nonspecific staining of 28S RNA (Fig. 3B) could have obscured weak signals. These results are consistent with studies with affinity-purified antibodies to β_1, which examined the tissue-specific distribution and the developmental time course of β_1 subunit expression (*7*).

To determine whether the β_1 subunit affected the function of the Na^+ channel α subunit, we injected *Xenopus* oocytes with in vitro–transcribed RNA encoding the Type IIA Na^+ channel α subunit alone or with RNA encoding the β_1 subunit. The macroscopic kinetics of Na^+ channel inactivation were accelerated dramatically in oocytes coexpressing the β_1 subunit (Fig. 4A), with $\tau = 6.27 \pm 1.24$ ms for α alone ($n = 6$) and $\tau = 1.15 \pm 0.14$ ms for α plus β_1 ($n = 7$). These results are similar to those for coexpression of Type IIA α subunit RNA and low molecular weight RNA from brain (*6*). Steady-state fast inactivation was shifted approximately 19 mV in the hyperpolarizing direction in oocytes coexpressing the β_1 subunit, with half-maximal inactivation values ($V_{1/2}$) of -26.1 ± 1.1 mV for α alone versus -44.5 ± 2.0 mV for α plus β_1 (Fig. 4B). Krafte and co-workers (*12*) observed a comparable difference between oocytes expressing the Type IIA Na^+ channel (−27.6 mV) and those expressing polyadenylated mRNA from rat brain (−47.5 mV); coinjection of low molecular weight brain RNA shifted the $V_{1/2}$ to a value comparable to that seen in Na^+ channels expressed by polyadenylated mRNA from rat brain. Coexpression of β_1 increased the peak current amplitude approximately 2.5 times, from -952 ± 211 nA for the α subunit alone to -2432 ± 767 nA for α plus β_1 (Fig. 4C). This increase in the peak current amplitude is similar to the 3.8-fold increase that was seen when low molecular weight rat brain polyadenylated mRNA was coinjected with RNA encoding the Type IIA α subunit (*6*).

Coexpression of the mRNA that encodes Ca^{2+} channel $\alpha_2\delta$ subunits with the α_1 subunit of the cardiac Ca^{2+} channel in

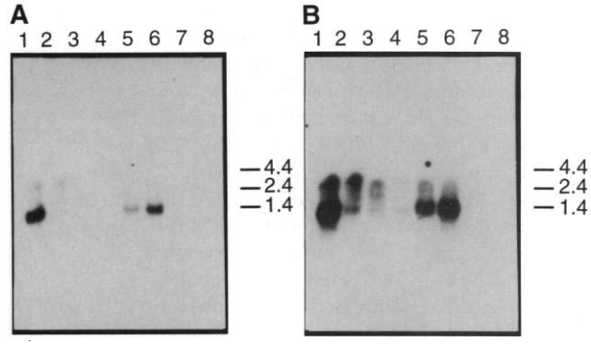

Fig. 3. Northern blot analysis of total RNA from various tissues with a β_1 subunit cRNA probe. Total RNA was prepared from the indicated tissues and analyzed by gel electrophoresis and Northern blotting (*24*). Lane 1, rat brain RNA; lane 2, 3-day-postnatal rat brain RNA; lane 3, rat liver RNA; lane 4, rat skeletal muscle RNA; lane 5, rat heart RNA; lane 6, rat spinal cord RNA; lane 7, eel electroplax RNA; lane 8, eel brain RNA. (**A**) Exposure 5 hours. (**B**) Exposure 17 hours. The second RNA band revealed after long exposure in (B) is nonspecifically labeled 28*S* RNA.

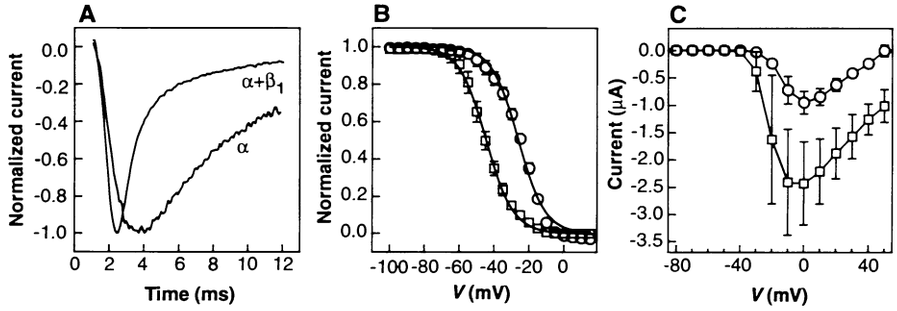

Fig. 4. The effects of coexpression of the β_1 subunit with the Type IIA Na^+ channel α subunit in *Xenopus* oocytes. Oocytes were injected with either 0.25 ng of in vitro–transcribed Type IIA α subunit RNA or coinjected with 0.25 ng of α subunit RNA and 5 ng of pβ1.C1Aa RNA. Na^+ channels were analyzed by two-electrode voltage clamping 2 days later. An excess of β_1 subunit RNA was required for the maximum effects illustrated here. The β_1 subunit we have cloned may preferentially interact with Type I or Type III α subunits, β_2 subunits may be required for efficient interaction of α and β_1 subunits, or the translational efficiency of the β_1 subunit RNA may be low. Preliminary experiments with β_1 subunit RNA from which 5′ untranslated regions containing predicted stem loop structures were removed gave improved expression suggesting a low translational efficiency of the natural β_1 mRNA. (**A**) The effect of the β_1 subunit on the Na^+ channel macroscopic current time course. Currents were evoked by step depolarizations to −10 mV from a holding potential of −100 mV. (**B**) Steady-state fast inactivation for Na^+ channels expressed from the α subunit alone or the α plus β_1 subunits. The 50-ms prepulses were followed by a test pulse to −5 mV. Peak current elicited during the test pulse is plotted as a function of prepulse potential for α subunit alone (○) or α plus β_1 (□). Means ± SD; solid lines represent fits to the means with a two-state Boltzmann distribution. The $V_{1/2}$ and slope factors are -26.1 ± 1.1 mV, 7.7 ± 0.8 mV per *e*-fold change ($n = 6$) for α subunit alone, and -44.5 ± 2.0 mV, 7.8 ± 0.7 mV per *e*-fold change ($n = 7$) for α plus β_1. (**C**) Current-voltage relations for Na^+ channels expressed from the α subunit alone or α plus β_1. Peak currents elicited by 12-ms voltage steps from a holding potential of −100 mV are plotted as a function of test pulse potential. Means ± SD for α subunit alone (○, $n = 4$) or α plus β_1 (□, $n = 5$). Procedures for in vitro transcription, oocyte injection, and electrophysiological recording are as described (*6, 28*). The bath recording solution consisted of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.5), and all voltage clamping was carried out at room temperature. For the experiments in (A) and (C), the resistive and capacitive current components were eliminated by subtraction of currents elicited by identical pulse protocols in the presence of 400 nM tetrodotoxin.

Xenopus oocytes causes increased peak current, more rapid inactivation, and inactivation at more negative membrane potentials (*25*), as we have observed for coexpression of β_1 subunits with Na^+ channel α subunits. The structural similarity between the Na^+ channel β_1 subunit and the Ca^{2+} channel δ subunit suggests that they may act by a similar mechanism and that the Ca^{2+} channel δ subunit may be responsible for the effects of the $\alpha_2\delta$ mRNA.

Our results define the primary structure of the β_1 subunit of the rat brain Na^+ channel, establish that it represents a new class of ion channel subunits, and show that it modulates functional expression of the α subunit. All three effects of low molecular weight brain mRNA on Na^+ channel expression and function (*6*, *12*) can be mimicked by coexpression with β_1 subunits. These results indicate that the β_1 subunit may be responsible for all three of the effects of low molecular weight brain mRNA on Na^+ channel expression and function. Although Na^+ channel α subunits expressed alone in mammalian cells inactivate rapidly, their level of functional expression is low relative to mRNA levels (*11*). Dissociation of β_1 subunits from purified rat brain Na^+ channels is accompanied by loss of function (*26*), and association with β subunits correlates with appearance of newly synthesized α subunits on the cell surface in neurons cultured from rat brain (*27*). Thus, the amount of expression and functional properties of Na^+ channels in brain neurons and other excitable cells may depend not only on the expression and properties of the α subunit but also on the expression and functional interaction with the β_1 subunit, as we have demonstrated directly here for expression in *Xenopus* oocytes. The multiple forms of the β_1 subunit observed in studies with subunit-specific antibodies (*7*) may differentially modulate Na^+ channel expression and function. The availability of cDNA clones encoding β_1 subunits will allow the molecular mechanisms of their interaction with α subunits and their modulation of expression and function of α subunits to be probed in detail.

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13. We purified Na^+ channels using the binding of [^{3}H]saxitoxin as an assay [R. P. Hartshorne and W. A. Catterall, *J Biol. Chem.* **259**, 1667 (1984)], and β_1 subunits were isolated by Triton X-114 phase extraction [B. F. X. Reber and W. A. Catterall, *J. Biol. Chem* **262**, 11369 (1987)] followed by SDS-PAGE and electroblotted to Immobilon PVDF membranes according to the method of P. Matsudaira (*ibid.*, p. 10035) The NH_2-terminal sequence was determined with an Applied Biosystems model 477A protein microsequencer with an on-line 120A phenylthiohydantoin analyzer for amino acid detection To generate peptide fragments, β_1 subunits were electroblotted to nitrocellulose, detected by staining with Ponceau S, and digested in situ with trypsin, endoproteinase Asp-N, or V8 protease [R H. Aebersold, J. Leavitt, R. A. Saavedra, L. E. Hood, S. B. Kent, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6970 (1987)] Cyanogen bromide fragments were generated from β_1 subunits prepared by SDS-PAGE followed by electroelution The cleavage fragments were separated by high-performance liquid chromatography (HPLC) and subjected to amino acid sequencing. For detection of the α and β_1 subunits by immunoblotting, SDS-PAGE was carried out according to U. K. Laemmli [*Nature* **227**, 680 (1970)] under reducing conditions in 10% polyacrylamide gels. Electroblotting to nitrocellulose and immunostaining with ^{125}I-labeled protein A were carried out as described by K. S. De Jongh, D K Merrick, and W. A Catterall [*Proc. Natl. Acad. Sci. U S.A.* **86**, 8585 (1989)].
14. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe, G, Gly, H, His; I, Ile; K, Lys; L, Leu, M, Met, N, Asn; P, Pro, Q, Gln, R, Arg, S, Ser, T, Thr; V, Val; W, Trp; and Y, Tyr
15. Synthetic peptides were synthesized corresponding to residues 1 to 18 of β_1 (with glutamate substituted at position 2 and norleucine at position 15) and residues 1491 to 1508 of Type IIA Na^+ channel α subunits with NH_2-terminal lysine and tyrosine. Antisera against HPLC-purified peptides were prepared in rabbits [D. Gordon *et al.*, *Proc Natl Acad. Sci. U.S.A* **84**, 8682 (1987); D. Gordon, D. Merrick, D A. Wollner, W. A. Catterall, *Biochemistry* **27**, 7032 (1988)]
16. C. C. Lee *et al*, *Science* **239**, 1288 (1988). A 256-fold degenerate sense oligonucleotide encoding the first six NH_2-terminal amino acids, as determined by protein sequencing (GGNGARGTNGARGTNGA), and a 256-fold degenerate antisense oligonucleotide corresponding to residues 29 to 34, as determined by protein sequencing (GTYTCNGCNGTNGTYTC), were used to prime a PCR with cDNA made from total rat brain RNA as template. The PCR was carried out in the presence of 1 μg of cDNA, 4 μM each primer, 10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgSO_4$, 0 001% (w/v) gelatin, 200 μM each deoxyribonucleotide triphosphate (dNTP), and 2.5 units of Taq DNA polymerase (Promega, Madison, WI). The 40 cycles of amplification were performed as follows: 3 min at 94°C, 2 min at 45°C, 4 min at 72°C. This was followed by a 10-min extension at 72°C and stopping of the reaction at 4°C. The resulting PCR products were treated with 8 units of T4 DNA polymerase (Promega, Madison, WI) for 5 min on ice, 5 min at room temperature, and then 15 min at 37°C in the presence of 100 μM each dNTP, 50 mM tris, pH 8, 5 mM $MgCl_2$, and 5 mM dithiothreitol, and separated on a 4% NuSieve gel (FMC BioProducts, Rockland, ME). A 102-bp band was excised from the gel and blunt-end ligated in the agarose into pBluescript SK$^+$ (Stratagene, La Jolla, CA). We identified the correct PCR product by screening the resulting bacterial colonies with a radiolabeled 36-fold degenerate oligonucleotide encoding residues 15 through 20, as determined by protein sequencing (ATGACNTTY-AAYATHCT). This 102-bp insert was sequenced with the Sequenase DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, OH) and end-labeled for use in subsequent library screening. Abbreviations in nucleotide sequences are N = A, G, C, or T, R = A or G, Y = C or T; and H = A, T, or C.
17. An oligo(dT)-primed rat brain cDNA library in the λ ZAP II vector (Stratagene, La Jolla, CA) was used.
18. Double-stranded DNA purified from clones, pBeta1 C1Aa and pBeta1.22A, in pBluescript SK was sequenced in both directions with the Sequenase DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, OH). DNA sequencing reactions were primed with the M13 Universal or Reverse primers or with β_1 subunit–specific primers.
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24. Total RNA from various rat or *Electrophorus electricus* tissues was isolated according to the method of V. Glisin *et al.* [*Biochemistry* **13**, 2633 (1973)] Each 20-μg sample was electrophoresed in a 1% agarose–formaldehyde (2.2 M) denaturing gel and transferred to nitrocellulose filters in 20× SSC (3M NaCl, 0.3 M sodium citrate · $2H_2O$). After baking under vacuum, the blot was prehybridized in 50 mM tris-HCl, pH 7 5, 6× SSC, 10× Denhardt's, 50% formamide, 0.5% SDS, 0.5 mg of sheared salmon sperm DNA per milliliter for 2 hours at 42°C, and then hybridized for 16 hours at 42°C in the same solution with 5 × 10^5 cpm of ^{32}P-labeled antisense cRNA per milliliter synthesized from pβ1.C1Aa. The nitrocellulose was washed twice at room temperature in 0.2× SSC, 0.5% SDS, then washed twice at 72°C in 0.1× SSC, 0.5% SDS for 2 hours. The filters were exposed to XAR-5 film at −80°C with one intensifying screen.
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