Cloning, Localization, and Functional Expression of Sodium Channel β1A Subunits

(Received for publication, September 7, 1999, and in revised form, October 7, 1999)

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Auxiliary β1 subunits of voltage-gated sodium channels have been shown to be cell adhesion molecules of the Ig superfamily. Co-expression of α and β1 subunits modulates channel gating as well as plasma membrane expression levels. We have cloned, sequenced, and expressed a splice variant of β1, termed β1A, that results from an apparent intron retention event. β1 and β1A are structurally homologous proteins with type I membrane topology; however, they contain little to no amino acid homology beyond the shared Ig loop region. β1A mRNA expression is developmentally regulated in rat brain such that it is complementary to β1. β1A mRNA is expressed during embryonic development, and then its expression becomes undetectable after birth, concomitant with the onset of β1 expression. In contrast, β1A mRNA is expressed in adult adrenal gland and heart. Western blot analysis revealed β1A protein expression in heart, skeletal muscle, and adrenal gland but not in adult brain or spinal cord. Immunocytochemical analysis of β1A expression revealed selective expression in brain and spinal cord neurons, with high expression in heart and all dorsal root ganglia neurons. Co-expression of αIIA and β1A subunits in Chinese hamster lung (CHL)1610 cells results in a 2.5-fold increase in sodium current density compared with cells expressing αIIA alone. This increase in current density reflected two effects of β1A: 1) an increase in the proportion of cells expressing detectable sodium currents and 2) an increase in the level of functional sodium channels in expressing cells.

[3H]Saxitoxin binding analysis revealed a 4-fold increase in Bmax with no change in Kp in cells coexpressing αIIA and β1A compared with cells expressing αIIA alone. β1A-expressing cell lines also revealed subtle differences in sodium channel activation and inactivation. These effects of β1A subunits on sodium channel function may be physiologically important events in the development of excitable cells.

Sodium channels isolated from brain are composed of a central pore-forming α subunit and two auxiliary subunits, β1 and β2, which do not form the pore yet play critical roles in channel modulation and expression. A mutation in the β1 gene (SCN1B) has been implicated to play a role in febrile seizures and generalized epilepsy, GEFS+ (1). The primary structure of the β1 subunit predicts an integral membrane glycoprotein with type I transmembrane topology as well as an extracellular Ig fold (2, 3). β1 subunits can be classified as members of the V-set of the Ig superfamily, which includes many cell adhesion molecules. β1 and α subunit co-expression has been well characterized in Xenopus oocytes and in mammalian cells. In oocytes, co-expression of type IIA (SCN2A) or μ1 (SCN4A) α subunits with β1 increases the proportion of sodium channels that function in a fast gating mode, accelerates the macroscopic rates of activation and inactivation, shifts the voltage dependence of inactivation in the hyperpolarizing direction, and increases the peak current amplitude consistent with increases in channel expression (4–9). In Chinese hamster lung (CHL)1 cells, stable coexpression of β1 with αIIA results in increased channel expression levels at the plasma membrane as well as moderate hyperpolarizing shifts in the voltage dependence of channel activation and inactivation (10).

β1 mRNA is expressed only after birth in the developing brain (5, 11). However, previous studies showing the developmental time course of β1 protein expression in rat forebrain suggested that multiple β1 subunit isoforms may be present (12). A 26-kDa β1-immunoreactive protein was observed at embryonic day 18. This protein was also expressed in adult adrenal gland, heart, skeletal muscle, and PC12 cells. After birth, there was a dramatic decrease in the level of this protein in brain, and little if any remained by postnatal day 14. The expression time course of this immunoreactive protein was complementary to that of β1 mRNA. Day 18 embryonic brain membranes also exhibited a low level of an immunoreactive peptide that migrated with an apparent molecular mass greater than 42 kDa. This protein was not detected in rat brain after birth. Other excitable tissues expressed multiple size forms of immunoreactive β1-like subunits as well. Adult rat heart and skeletal muscle membrane preparations exhibited 38- and 41-kDa bands on Western blots in addition to the 26-kDa band. The 41-kDa immunoreactive band observed in these studies was shown to be the adult rat brain isoform and was later identified as C1αa.β1 (4). The immunoreactive peptides identified in the previous study were detected with a polyclonal antibody raised against purified β1 subunits; thus, they could represent β1 subunit isoforms that contained signif-

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ically different mRNA sequences from C1Aa,β1 that may not have been detected using previous methods.

To test this hypothesis, we screened a rat adrenal cDNA library with a probe encoding only the coding region of β1. The present study reports the molecular cloning and functional expression of β1A, a splice variant of the β1 gene that contains identical amino-terminal and extracellular Ig fold regions as β1 followed by a significantly different extracellular juxtamembrane domain, predicted transmembrane region, and predicted intracellular COOH-terminal domain. β1A mRNA is expressed early in embryonic brain development and then disappears after birth. Western blot analysis of membrane preparations using an antibody to a unique, extracellular region of β1A not found in β1 showed that β1A protein is expressed in adult rat heart, skeletal muscle, and adrenal gland but was not detected in adult rat brain or spinal cord. Immunochemical analysis of β1A expression in adult rat tissues revealed high expression in heart and dorsal root ganglion and selective expression in some areas of the brain and spinal cord. β1A functions to increase channel expression at the plasma membrane when coexpressed with α1IA subunits in CHL fibroblasts. Unlike β1, however, mean steady state inactivation curves for α1A-expressing cell lines were shifted to more positive potentials than the mean inactivation curves for cells expressing α alone. Previous studies showed that coexpression of α1 and β1 subunits in CHL cells shifted the voltage dependence of inactivation to more negative potentials compared with α alone (10). Therefore, the novel, carboxyl-terminal domains of β1A may be important for electrophysiological function. It has been shown previously that the extracellular domain of β1 is essential for expression and function of the αβ1 complex in Xenopus oocytes (13, 14). We propose that the extracellular Ig fold, common to β1 and β1A, is essential for the observed increases in channel expression levels. Thus, this report introduces a novel splice variant of β1, β1A, and adds to our understanding of β1 structure-function relationships in terms of channel expression levels and electrophysiology.

**EXPERIMENTAL PROCEDURES**

**Library Screening**—A cDNA probe encoding nucleotides 345–911 of pβ1C1Aa (4) was labeled with digoxigenin following the manufacturer’s instructions (Roche Molecular Biochemicals) and used to screen a λ Zap Express rat adrenal cDNA library prepared by Stratagene (La Jolla, CA). pBK plasmids containing cDNA inserts that hybridized strongly to the probe were rescued from the λ phage according to the manufacturer’s instructions, confirmed by Southern blot analysis, and sequenced using ThermoSequenase (Amersham Pharmacia Biotech).

**Reverse Transciptase-PCR from Rat Adrenal RNA**—To confirm independently that the β1A transcript identified by library screening was expressed by rat adrenal gland, we amplified a region of β1A from the amino terminus past the region in which the amino acid sequence changed from identity to nonidentity to β1, or the putative splice site, by reverse transcriptase-PCR using rat adrenal gland total RNA as template and β1A-3 and β1A-1 as primers (5′-GAGGATGACCCGCTTGGAGG-3′, primer sequence common to β1 and β1A) and β1A-5 and β1A-1 (5′-GAGGACACAGCAACGC-3′, primer sequence unique to β1A) as oligonucleotide forward and reverse primers, respectively. Rat adrenal gland cDNA was synthesized from total RNA using Superscript II (Life Technologies, Inc.) according to the manufacturer’s instructions in a total volume of 20 µl. 2.3 µg of total rat adrenal RNA (purified using Trizol reagent; Life Technologies) was used in the reaction. The PCR conditions were as follows: 1 µl of cDNA, 0.5 µM concentration of each primer, 200 µM concentration of each dNTP (Roche Molecular Biochemicals), 5 µl of MgCl2-free 10× PCR buffer (Perkin-Elmer), and 1.5 µM MgCl2 were mixed in a total volume of 50 µl. Following a hot start at 94 °C, 0.25 µl of AmpliTaq DNA polymerase (Perkin-Elmer) was added to the reaction, and the amplification cycle was started. The cycling parameters were as follows: 40 cycles of 45 s at 94 °C, 20 s at 60 °C, 90 s at 72 °C. This was followed by 10 min at 72 °C and then 4 °C until the tubes were removed from the thermocycler (GeneAmp 2400, Perkin-Elmer). Analysis of the PCR products on a 1% agarose gel revealed a 750-base pair band (data not shown). The band was excised from the gel, subcloned into pcCR2.1 (Invitrogen, Carlsbad, CA), and analyzed using ThermoSequenase (Amersham Pharmacia Biotech). The sequence obtained from this PCR clone was identical to that obtained from the original β1A clone plaque-purified from the adrenal cDNA library.

**RNAi Gene—**Intron 3 of the rat β1 gene (15) was amplified by PCR using rat genomic DNA as template, and oligonucleotides that encode β1P coding sequence flanking intron 3, VVDR (5′-AGATCCACCTGGAGG-TGGTGCACAAGC-3′) and ARND (5′-ACACAGTGATGCTCATCT-CTGGTG-3′), as forward and reverse primer, respectively; and the Expand Long Template PCR System (Roche Molecular Biochemicals). All oligonucleotide primers were synthesized by Life Technologies. The amplification conditions were as follows: 300 ng of rat genomic DNA (CLONTECH Laboratories, Inc., Palo Alto, CA), 250 ng of each primer, 1 µmol concentration of each dNTP (Roche Molecular Biochemicals), and 5 µl of Expand Buffer 3 were mixed in a total reaction volume of 25 µl. Following a hot start at 95 °C, 0.5 µl of Expand DNA polymerase were added, and the amplification cycle was started. 40 cycles of the following regimen were performed: 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 4 min plus 20 s added to each successive cycle. The samples were then held at 4 °C until removal from the thermocycler (GeneAmp 2400, Perkin-Elmer). The 5-kilobase pair PCR product (data not shown) was gel-purified and sequenced directly using oligonucleotide VVDR as the sequencing primer.

**RNAi Protection Analysis**—A plasmid containing a RNase protection template (pRPA-1) was constructed as described (1). Oligonucleotides 364–533 in the β1A sequence. Briefly, a 169-nucleotide AluI/AvelI fragment was excised from pBK,β1A and ligated into the Smal and AccI sites of Bluescript (Stratagene). The resulting plasmid was then sequenced using ThermoSequenase. To synthesize labeled cRNA, a 10-µg aliquot of pRPA-1 was linearized with XhoI, ethanol-precipitated, re-suspended in RNase-free water, and labeled with digoxigenin using the T7 MAXiScript kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Following a 2-h incubation at 37 °C, the reaction was incubated at 95 °C for 2 min, chilled on ice, and then treated with RNase-free DNase (2 units) for 15 min at 37 °C. EDTA (final concentration 30 mM) was added to stop the reaction. Free nucleotides were removed by ethanol precipitation with 0.5 µl ammonium acetate, and the final pellet was resuspended in 20 µl RNase-free water. The probe (RPA-1) was quantitated by comparison of serial dilutions of the labeled probe with serial dilutions of control digoxigenin-labeled RNA following the manufacturer’s instructions. Typical labeled probe concentrations were 10 ng/µl throughout our experiments.

**RNase protection experiments were performed using the HybSpeed RPA kit (Ambion). Briefly, 20 µg of rat embryonic day 18 brain RNA was hybridized with 1 µl of digoxigenin-labeled RPA-1 probe and 30 µg of yeast tRNA in 0.5 µl ammonium acetate plus 2.5 volumes of ethanol. The reaction tubes were left at −20 °C for 15 min, and the RNA was precipitated by centrifugation in a microcentrifuge at top speed. The RNA was resuspended in 10 µl of HybSpeed hybridization buffer that had been preheated to 95 °C and vortexed vigorously, and the tubes were placed at 95 °C for 3 min. The samples were then hybridized for 10 min at 95 °C in a mixture of 10 units/ml RNase A and 400 units/ml RNase T1) for 30 min at 37 °C. 150 µl of HybSpeed Inactivation/Precipitation mix were added to each reaction, and the RNA was precipitated and resuspended in 10 µl of gel loading buffer 1. The reactions were separated on a 1.5-mm-thick 6% acrylamide TBE denaturing gel containing 7% urea in the Mini-Protean gel format (Bio-Rad), transferred to nylon (Roche Molecular Biochemicals), and UV-cross-linked using a Stratalinker (Stratagene). Hybridization of the digoxigenin-labeled probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:10,000 dilution) and CSPD chemiluminescent substrate solution (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

**Preparation of RNA and Northern Blot Analysis**—Time-mated pregnant female Harlan Sprague Dawley rats were anesthetized with 60 mg/kg Beuthanasia-D intraperitoneal (Schering-Plow Animal Health Corp., Kenilworth, NJ), and the fetuses were surgically removed. Birthing day 9 rats were homogenized in their entirety in Trizol reagent (Life Technologies) to purify total RNA according to the manufacturer’s instructions. Whole fetal brains were dissected at the remaining embryonic time points, and total RNA was purified using Trizol reagent. RNA samples were subsequently purified from the adrenal glands of the adult female rats. Postnatal rats at the indicated ages were anesthetized with Beuthanasia-D, brains were dissected, and total RNA was purified with Trizol reagent. Northern blot analysis of 20 µg of each sample was performed as described previously using a digoxigenin-labeled β1A antisense cRNA probe encoding nucleotides 428–850 or a digoxigenin-labeled antisense cRNA probe specific to the 3′-untranslated region of β1A (4). The labeled RNA was detected using a chemiluminescent substrate solution (Roche Molecular Biochemicals). The membranes were then exposed to X-ray film (RTO 45, Dupont).
translated region of β1 (nucleotides 1053–1508 of pBl1.C1Aa (4).

**Construction of βIA Expression Vector**—A plasmid containing β1 cDNA including an in-frame amino-terminal hemagglutinin (HA) epitope tag was obtained as a generous gift from the laboratory of R. A. Maue (Dartmouth University) (16). This construct has been shown to express subunits in Xenopus oocytes. The HA-tagged β1 cDNA was recloned into the EcoRI and NotI sites of the mammalian expression vector pCIneo (Promega, Madison, WI) to create pCl.β1-HA. pCl.β1-HA was subsequently digested with Accl and NotI and agarose gel-purified to remove the 3′ end of β1. The Accl restriction endonuclease site is common to β1 and βIA. pBlK.β1A cDNA was digested with Accl and NotI and gel-purified. The 3′ end of Accl was then ligated to βIA/NotI-digested pCl.β1-IA to create pCl.β1A-IA. The junctions were then sequenced to confirm that the segments of β1 and βIA were successfully ligated in frame.

**Transfection of SNαIIA Cells with HA-tagged β1A—SNαIIA cells were transfected with pCl.β1A-IA using DOTAP as described previously (10). Because SNαIIA cells are resistant to G418 as a result of the original transfection of the β1A subunit, pCl.β1A-IA was cotransfected with pSV2+Hog to confer resistance to the antibiotic hygromycin. Drug selection with hygromycin (400 μg/ml) required approximately 1 week; clonal cell lines were selected, analyzed by Northern blot, and expanded for further analysis.

**[3H]Saxitoxin Binding Analysis**—Whole cell saturation binding analysis was performed as described previously (10) over a concentration range of 0.1-10 μM [3H]saxitoxin (([3H]STX) with the addition of 10 μM unlabeled tetrodotoxin (TTX; Calbiochem) to assess nonspecific binding. ([3H]STX (28 Ci/mmol) was obtained from Amersham. Binding data were normalized to protein concentration using the BCA Protein Assay kit (Pierce). Saturation binding data were analyzed by nonlinear regression using Prism (GraphPad Software, La Jolla, CA) to obtain K_D and R_max values.

**Antibodies**—A multiple antigenic peptide with amino acid sequence RWRDVRKEDGRDLVSHRGGQ, encoded by nucleotides 160–177 of β1A, was synthesized by the Protein and Carbohydrate Structure Facility at the University of Michigan. Rabbit polyclonal antibodies were subsequently generated in two separate animals and tested by enzyme-linked immunosorbent assay against the β1A peptide to determine the antibody titer (Research Genetics, Inc., Huntsville, AL).

**Western Blot Analysis of β1A Protein Expression**—Adult female Harlan Sprague Dawley rats were sacrificed by decapitation. Brain, spinal cord, heart, skeletal muscle, and adrenal gland tissues were immediately removed, minced, and briefly stored on ice. SNαβ1A-16 cell line cells were washed with PBS and scraped into 50-ml conical tubes. Membranes were prepared as described previously (10), and the final pellets were resuspended in 50 mM Tris, pH 8, 10 mM EGTA containing Complete-Mini protease inhibitor tablets according to the manufacturer's instructions (Roche Molecular Biochemicals). The total protein in each membrane preparation was quantitated with the BCA Protein Assay kit (Pierce) using bovine serum albumin as the standard. 250 μg of each membrane preparation were separated by SDS-PAGE as described (18), transferred to nitrocellulose (HyBond ECL, Amersham Pharmacia Biotech), and stained with Ponceau-S prior to immunodetection. Western blot analysis was performed as follows. The blot was washed for 10 min in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature and then blocked for 1 h in 5% nonfat dry milk in TBS-T at room temperature. Primary antibody (a 1:750 dilution) was applied in blocking solution for 30 min at room temperature. The blot was then washed five times for 15 min each in TBS-T. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, ICN) diluted to 1:100,000 in blocking solution was applied for 30 min at room temperature. The blot was then washed five times for 15 min each in TBS-T. SuperSignal WestFemto chemiluminescent substrate solution (Pierce) was applied according to the manufacturer's instructions, and the blot was placed between plastic sheet protectors and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech) for the indicated times (typically 10–30 s) at room temperature.

**Immunohistochemical Analysis of β1A Expression**—Sprague Dawley rats (4–6 weeks of age; Harlan Industries, Indianapolis, IN) were perfused with 4% neutral buffered formalin for approximately 20 min, and tissues were removed for further processing. Tissues were pierced overnight in 10% neutral buffered formalin, processed, embedded in paraffin blocks, and sectioned onto slides (5-μm thickness). Tissues were processed for immunohistochemistry as described previously (17). Briefly, slides were blocked with normal goat serum, incubated with rabbit anti-rat β1A antibody at a dilution of 1:600 and then incubated with biotinylated goat anti-rabbit IgG (Vector Labs, Burlington, CA). All incubation were performed for 30 min at room temperature. Detection was accomplished using the ABC-horseradish peroxidase system (Vector Labs) followed by 3'-diaminobenzidine (Biomedica, Foster City, CA) as the chromogen, stained in Mayer's hematoxylin, and covered-slipped with Permount (Fisher).

**Electrophysiological Analysis**—Electrophysiological recordings on SNαIIA and SNαβ1AIA cells were performed by the patch clamp technique in the whole cell configuration (18), using an Axopatch 200B patch clamp amplifier and pCLAMP software (Axon Instruments, Foster City, CA). Data were filtered at 5 kHz and digitally sampled at 50 kHz. Series resistance was compensated 60–80%. Capacitative transients, elicited by voltage steps, were partially canceled using the inverting offset function. Additional subtraction of transients and leak currents was obtained using the P4 procedure (19). For whole cell recordings, recording pipettes were filled with 105 mM CsF, 10 mM CsCl, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.4, with CsOH. Pipette resistances were 1–3 MΩ. The bath solution consisted of 130 mM NaCl, 4 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES, pH 7.4, with NaOH. As described previously (10), the voltage dependence of sodium current activation and inactivation progressively shifted to more negative potentials over the first few minutes of experiments with fluoride-based intracellular solutions. Thus, all experiments were begun 10 min after break in, at which point the shifts in channel gating had stabilized.

For each cell, we examined the voltage dependence of current activation and inactivation. Activation was assessed by applying test pulses to potentials from −50 to +70 mV in 5-mV steps, following a 100-ms prepulse to −100 mV. Peak current amplitude (I_peak) was measured at each test potential and converted to conductance (g) according to the equation, g = I_peak/(V_rev − V_test), in which V_test is the test potential and V_rev is the current reversal potential, determined by linear extrapolation of the straight line portion of the falling phase of the current-voltage relationship. The conductance values were normalized with respect to the maximal conductance, plotted as a function of V_test and fit with the Boltzmann equation: I/I0 = 1 + (V/V0−k/h), in which V is the midpoint of the curve and k is a slope factor. Steady state inactivation was examined by applying 100-msec-long prepulses to potentials ranging from −100 to −10, in 5-mV steps, followed by a test pulse. Peak and steady-state currents evoked by the test pulses were normalized with respect to the largest currents plotted, as a function of prepulse potential, and fit with the Boltzman equation.

**RESULTS**

**Molecular Cloning and Analysis of β1A**—A rat adrenal gland cDNA library prepared in the A Zap Express vector was screened with a digoxigenin-labeled cDNA probe encoding nucleotides 345–911 of pBl1.C1Aa (4). A clone encoding a protein with a 5′ region of identity to β1 and a novel 3′ region was identified by DNA sequencing. The identity of this clone was then confirmed independently by reverse transcriptase-PCR from rat adrenal cDNA using the oligonucleotides β1A-3 and β1A-5 followed by DNA sequencing, as described under “Experimental Procedures.” This clone, designated β1A, encoded a novel 253-amino acid protein of 29,055 daltons (predicted molecular mass of the mature protein with the signal sequence removed), which contains a predicted amino-terminal region of identity to β1, residues Met1 through Lys120, followed by a novel carboxyl-terminal region (Fig. 1. A and B). Hydrophylicity analysis of the novel, carboxyl-terminal region revealed an apparent 66-amino acid extension of the extracellular region of β1 followed by a 19-amino acid transmembrane domain and short, 39-amino acid intracellular carboxyl terminus (Fig. 1. A and C). The novel 3′ region of β1A is structurally homologous to β1 in that it predicts a transmembrane domain and short intracellular region, yet it contains little to no homology at the amino acid level (Fig. 1B). Interestingly, the amino-terminal region common to β1 and β1A contains the extracellular immunoglobulin fold. β1A can thus be characterized structurally as a cell adhesion molecule (17).

Analysis of the novel 3′ region of β1A by BLAST-P search of the Swiss-Prot data base revealed a 55-residue region of β1A with 32% identity to an extracellular low density lipoprotein receptor class A domain of human low density lipoprotein receptor-related protein 2 (LRP2), also called megalin or glyco-
protein 330 (Fig. 1) (20–23). This region of homology in β1A is predicted to be located extracellularly, just proximal to the plasma membrane followed by the transmembrane region. LRP2 has been shown to be a cysteine-rich type I membrane protein that forms a multimeric complex with receptor-associated protein. LRP2 binds clusterin with high affinity and is localized to clathrin-coated pits, suggesting that it may be an endocytic receptor. Interestingly, LRP2 has been shown to interact with extracellular matrix components, similar to sodium channel β1 and β2 subunits (24). The BLAST-P search also revealed a 63-residue region of β1A with 26% identity to tensin, a protein that has been implicated as the anchor for actin filaments at focal adhesions and is thought to act as a linker between the cytoskeleton and signal transduction proteins (25). The region of homology to β1A is located in the insertion domain of tensin. This domain has been shown to permit polymerization of actin filaments.

β1A Is Encoded by a Retained Intron in the β1 Gene—The genomic organization of the human sodium channel β1 subunit gene has been reported previously (15). Using this information, we determined that the site of divergence between the β1 and β1A cDNAs was located precisely at the exon 3-intron 3 boundary of the β1 gene (Fig. 1E). Furthermore, a consensus sequence for exon-intron boundaries in genomic DNA was readily identified at this location. Amplification of intron 3 (approximately 5 kilobase pairs; data not shown) from rat genomic DNA by PCR followed by sequencing showed that the sequence of β1A beyond the amino acid sequence VVDK was indeed that of intron 3. We next performed a series of RNase protection experiments using a probe that was designed to span the exon 3-intron 3 boundary in the rat sequence. Fig. 2 shows that this 169-nucleotide probe was fully protected by rat embryonic day 18 brain mRNA. Thus, we propose that the novel extracellular, transmembrane, and carboxyl-terminal domains of β1A are encoded by alternative splicing of a retained intron within the β1 gene that includes an in-frame termination codon. These data are in agreement with the previously reported observation that β1 is represented only once in the rat and human genomes (26, 27).

β1A mRNA Is Expressed in Embryonic Brain and Adult Adrenal Gland—A comparison of the developmental time courses of β1A and β1 mRNA expression in developing rat
brain was determined using specific, noncross-hybridizing antisense cRNA probes for β1A and β1, respectively. Fig. 3 compares the developmental time course of expression of β1A (upper panel) versus β1 (lower panel) in total rat brain RNA from embryonic day 9 through postnatal day 21. The transcript size of β1A is approximately 4.4 kilobase pairs and reflects the retention of a portion of intron 3. Interestingly, the expression time course of β1A parallels that of the 26-kDa β1-immunoreactive band described previously (12) and complements the expression pattern of β1 (5). Thus, β1A is expressed early in development and disappears after birth. In contrast, β1 expression is not detectable during embryonic brain development and becomes detectable as β1A mRNA expression is decreased.

**Analysis of β1A Protein Expression**—To determine whether alternative splicing of the β1 gene resulted in expression of a novel protein, a polyclonal antibody was generated against a multiple antigenic peptide containing the amino acid sequence RWDRWKEDGRLVSHRGQ encoded by the retained portion of intron 3 found in the β1A cDNA clone. Aliquots of membrane preparations from adult rat brain, heart, skeletal muscle, spinal cord, and adrenal gland were separated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose, and Western blot analysis was performed using the β1A antibody described above (1:750 dilution). As shown in Fig. 4, β1A immunoreactive bands migrating at approximately 45–50 kDa were observed in heart, skeletal muscle, and adrenal gland but were not detected in brain or spinal cord. An immunoreactive doublet was observed in adrenal gland. The absence of immunoreactive β1A protein bands in adult brain and spinal cord is consistent with the Northern blot results shown above.

**Immunohistochemical Analysis of β1A Expression**—Positive β1A protein immunoreactivity was detected as brown product (Figs. 5 and 6). Product was not detected in the negative controls, which included the replacement of the primary antibody with similar species isotype serum (data not shown). Fig. 5 represents various central and peripheral neuronal populations that contained β1A. Fig. 5, A and B, demonstrates that β1A was found in most but not all Purkinje cells in the cerebellum. In addition, some β1A-positive neurons in the dentate nucleus of the cerebellum were observed (Fig. 5C). β1A was absent from the granular layer (G) and the molecular layer (M) of the cerebellum. In Fig. 5D, a distinct population of pyramidal neurons of the cerebral cortex contained β1A, while glial cell populations remained negative. Spinal cord also contained several distinct populations of β1A-containing neurons. Fig. 5E shows β1A in a small population of motor neurons, while β1A
neurons were also observed in laminae II–V of the dorsal horn. All neuronal cell types of the dorsal root ganglia contained β1A (Fig. 5F), while processes and glial cells were negative for the β1A product.

Fig. 6, A–D, shows examples of nonneuronal sites of β1A localization. In Fig. 6A, the membranes of individual muscle fibers in rat atria were positive for β1A. Additionally, other areas of the rat heart such as ventricles contained β1A product (data not presented). β1A immunoreactivity was also observed in the alveoli (Fig. 6B) and in some bronchus columnar epithelial cells (Fig. 6C). Finally, labeling of endothelial cells in the pulmonary artery is shown in Fig. 6D.

Mammalian Cell Expression—To investigate the functional role of β1A, we constructed a HA epitope-tagged version of the β1A cDNA. We included the epitope tag for potential use in the event that our polyclonal antibody production was unsuccessful. We were successful in raising an anti-β1A antibody and thus did not use the HA tag in these experiments. We created stably transfected cell lines expressing β1A in the previously characterized SNAILA cell line. SNAILA cells are a stable line expressing type IIA sodium channel α subunits in CHL cells (10). Because SNAILA cells are G418-resistant, we co-transfected pcDNA3-β1A with pSV2⁺Hyg in a 10:1 ratio (pcDNA3-β1A:pSV2⁺Hyg) so that transfected clones could be selected with the antibiotic hygromycin. A number of hygromycin-resistant colonies were analyzed by Northern blot for β1A mRNA expression. Positive clones were expanded and analyzed further by [3H]STX binding. Western blot analysis of one of these cell lines, SNAILA/β1A-16, is shown in Fig. 4, in which an immunoreactive band of approximately 45 kDa was observed.

Fig. 4. Western blot analysis of β1A expression. Brain, spinal cord, heart, skeletal muscle, adrenal, and SNAILA/β1A-16 cell membrane preparations were prepared as described previously (10). The total protein in each membrane preparation was quantitated with the BCA protein assay kit using bovine serum albumin as the standard. 250 μg of each membrane preparation were separated by SDS-polyacrylamide gel electrophoresis as described previously (10), transferred to nitrocellulose, and stained with Ponceau-S prior to immunodetection. Western blot analysis was performed as follows. The blot was washed for 10 min in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature. Primary β1A antibody (1:750 dilution) was applied in blocking solution for 30 min at room temperature. The blot was then washed five times for 15 min each in TBS-T. Secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG, TCB) diluted to 1:100,000 in blocking solution was applied for 30 min at room temperature. The blot was then washed five times for 15 min each in TBS-T. SuperSignal WestPento chemiluminescent substrate solution was applied according to the manufacturer’s instructions, and the blot was placed between plastic sheet protectors and exposed to Hyperfilm-ECL for 10 s at room temperature. B, adult brain; H, adult heart; SM, adult skeletal muscle; A, adult adrenal; SC, adult spinal cord; TC, transfected cells (SNAILA/β1A-16).

[3H]STX binding analysis revealed a significant increase in the expression levels of functional sodium channels at the plasma membrane of SNAILA/β1A-16 cells as compared with the parental line, SNAILA (Table 1). Nonlinear regression analysis of saturation binding showed a 4.4-fold increase in Bmax as compared with SNAILA with no significant change in the Kd (0.8 nm for SNAILA versus 0.9 nm for SNAILA/β1A-16). Our results are similar to those of a previous study showing that coexpression of αIIA and β1 resulted in a 2–4-fold increase in the level of [3H]STX binding compared with cells expressing αIIA alone (10). The Kd values obtained in the present study were very similar to those reported values as well. Our data suggest that a function common to β1 and β1A is to increase the level of sodium channel expression at the plasma membrane. We hypothesize that β1 and β1A may stabilize the conformation of channels such that they become more resistant to degradation and/or target newly synthesized channels to the plasma membrane from intracellular stores. Because β1 and β1A contain a common cell adhesion molecule domain, we propose that the extracellular Ig loop may be necessary for this function.

To determine whether coexpression of β1A subunits affected the functional properties of type IIA sodium channels in CHL cells, we used whole cell electrophysiological recording to compare sodium currents in the parent SNAILA cell line and in three different SNAILA/β1A cell lines. Fig. 7A shows currents, elicited by depolarizations to varying test potentials, recorded in a typical SNAILA cell and a typical SNAILA/β1A cell. As is evident from these traces, coexpression of β1A did not dramatically alter the properties of voltage-activated sodium currents. Nevertheless, currents in β1A-expressing cell lines were subtly different from currents in SNAILA cells. For example, mean steady state inactivation curves for SNAILA/β1A cell lines were shifted to more positive potentials than the mean inactivation curve for SNAILA cells (Fig. 7, B and D). Although this difference was quite small, it was observed in all three SNAILA/β1A cell lines and was statistically significant in two of the three β1A-containing lines (SNAILA/β1A-7, p = 0.037; SNAILA/β1A-8, p = 0.024). Thus, one effect of β1A association with αIIA may be a small positive shift in the voltage dependence of steady state inactivation. For activation, mean voltage-conductance curves for two of the three β1A-expressing lines were statistically indistinguishable from SNAILA (Fig. 7, B and D); however, for SNAILA/β1A-16, the voltage dependence of activation was significantly more negative than for SNAILA (p = 0.001). Thus, data from one of the three β1A cell lines suggest that β1A may also alter sodium channel activation. For comparison, Fig. 7D also shows the effects of the adult β1 isoform on the voltage dependence of sodium channel activation and inactivation. As has been shown previously (10), when coexpressed with αIIA in CHL cells, β1 shifted the voltage dependence of steady state inactivation approximately 10 mV negative compared with αIIA alone (Fig. 7D). Thus, β1 and β1A have clearly different effects on steady state inactivation. SNAILA/β1A cells also exhibited a small negative shift in the voltage dependence of activation compared with SNAILA cells, as was previously reported (10).

To determine whether β1A affected the time course of macroscopic sodium currents, we fit the decaying phase of whole cell currents, elicited over a range of test potentials, with single exponential functions (Fig. 7C). For SNAILA, the inactivation time constants determined from these fits were progressively less at progressively more positive test potentials, approaching a minimum of approximately 0.5 ms at the most positive test potentials examined. Inactivation time constants were significantly less for SNAILA/β1, suggesting that β1 may slightly speed inactivation time course (Fig. 7C). For two of the three SNAILA/β1A cell lines, the rate of current inactivation was virtually identical to SNAILA (Fig. 7C). However, for SNAILA/β1A-16, inactivation was similar to SNAILA at all test potentials examined (Fig. 7C). Because sodium channel inactivation is coupled to activation (19, 28), it is likely that the faster decay rates for SNAILA/β1 and SNAILA/β1A-16 currents were, at least in part, secondary to the negative shifts in
activation that were observed in these two cell lines. In addition, it is also possible that β1 and β1A subunits have direct effects on the rate of sodium channel inactivation.

The most dramatic effect of β1A, detected electrophysiologically, was a large increase in the amplitudes of macroscopic sodium currents. This is illustrated in Fig. 8A, which shows current densities for depolarizations to +10 mV in SNaIIA, SNaIIAβ1A, and SNaIIAβ1 cell lines. Current densities for SNaIIA cells were 69 pA/picofarads, whereas current densities were approximately 2.5 times greater for the three SNaIIAβ1A cell lines (Fig. 8A). The increase in current density observed with coexpression of β1A was similar to that seen with coexpression of the adult β1 isoform (Fig. 8A). This increase in mean current density reflected two distinct effects of β1A on sodium channel expression. First, β1A greatly increased the proportion of cells with measurable whole cell sodium currents. This effect is shown in Fig. 8B, which plots the number of SNaIIA (black bars) or SNaIIAβ1A (white bars) cells with peak currents within different amplitude ranges. For SNaIIA, this amplitude-frequency distribution was bimodal. In 40% of the cells (16 of 40), currents were indistinguishable from the small inward currents recorded in untransfected CHL cells (i.e. <100 pA). In the remaining 60% of the cells, currents ranged from 500 pA to 5 nA and thus were clearly due to expression of cloned type IIA channels. In contrast, all SNaIIAβ1A cells expressed large sodium currents (Fig. 8A). The frequency histogram for SNaIIAβ1A followed a normal distribution with a modal current range of 2–3 nA. To determine whether the lower mean current density of SNaIIA cells was solely due to its large proportion of low expressing cells, we recalculated the mean current density for SNaIIA, excluding these low expressers. Eliminating low expressing cells increased the mean current density for SNaIIA cells from 69 to 116 pA/picofarads (Fig. 8A); however, this value was still significantly smaller than the mean current density of cells expressing β1A (p = 0.014). Thus, even when comparing only those cells that expressed measurable sodium currents, β1A still increased the density of functional sodium channels on the cell surface.

**DISCUSSION**

A number of cases of intron retention have been reported in the literature, including alternative splicing of the genes encoding leukocyte-common antigen-related protein tyrosine phosphatase, CD44, effector cell protease receptor-1, the microtubule-associated protein tau, thyrotropin-releasing hormone receptor, and bovine growth hormone (29–35). In many cases, the retained intron contains an alternate, in-frame termination codon as well as a polyadenylation signal. Alternative splicing that results in retention of the intron in the primary transcript thus results in an isofrom of the protein containing a novel carboxyl terminus. Interestingly, this is not the first report of

**FIG. 5.** Immunocytochemical analysis of β1A expression in neurons. A, × 10 magnification of the rat cerebellum shows β1A immunolabeling in both Purkinje cells (large arrow) and a population of neurons in the cerebellar white matter (small arrow). M, molecular layer; G, granular layer; W, white matter. B, × 60 magnification of the cerebellar Purkinje cell layer, demonstrating that most of the Purkinje cells contain β1A (black arrow), but some adjacent Purkinje cell neurons remain negative for β1A (blue arrow). C, ×60 magnification of the cerebellar dentate nucleus, indicating positive neurons (black arrow) and negative glial cells (blue arrow). D, β1A-positive pyramidal neurons in the rat cerebral cortex (black arrow) and negative glial cells (blue arrow). E, ×40 magnification of β1A-positive spinal cord motor neurons (black arrow). F, × 60 β1A-positive c-fiber neuron (black arrow) adjacent to β1A-positive large Aβ neurons.
cell adhesion molecule domain common to the two isoforms. The results of our \[^{[3H]}\)STX binding experiments support this hypothesis. We propose that interaction of the extracellular cell adhesion molecule domain (Ig fold) common to \(\beta 1\) and \(\beta 1A\) with \(\alpha\) may be responsible for the observed effects on channel expression levels. Consistent with this interpretation, two previous studies have shown that the extracellular domain of \(\beta 1\) is essential for modulation of both brain and skeletal muscle \(\alpha\) subunits, whereas the intracellular carboxyl-terminal domain is not; truncated \(\beta 1\) subunits lacking the intracellular carboxyl terminus are fully functional in terms of kinetic modulation of brain and skeletal muscle \(\alpha\) subunits expressed in \(Xenopus\) oocytes (13, 14). Residues predicted to be in the Ig fold of \(\beta 1\) interact with type IIA \(\alpha\) subunits (14). Thus, the extracellular cell adhesion domain common to \(\beta 1\) and \(\beta 1A\) appears to be required for function.

Sodium currents in \(\beta 1A\)-expressing cell lines also exhibited subtle functional differences compared with the parent \(SNaIIA\) cell line. For example, inactivation curves in \(SNaIIA/\beta 1A\) cell lines were shifted to slightly more positive potentials than inactivation curves for \(SNaIIA\) cells. In contrast as shown both here and in a previous study (10), coexpression of \(\beta 1\) with \(\alpha\) in \(CHL\) cells shifted inactivation to potentials approximately 10 mV more negative than for cells expressing \(\alpha\) alone. Perhaps the differences between these two \(\beta\) subunit isoforms located in the putative juxtamembrane and/or transmembrane domains are responsible for these subtle distinctions in functional effects. Makita et al. (38) reported that a \(\beta 1/\beta 2\) subunit chimeric construct containing the extracellular region plus the first 6 residues of the transmembrane domain of \(\beta 1\) was sufficient to modulate sodium channel skeletal muscle \(\alpha\) subunits expressed in oocytes. Interestingly, this construct included an additional segment of \(\beta 1\) (ANRDMASIVSEIMMYVL) that is located carboxyl-terminal to the intron 3 splice site and is therefore not present in \(\beta 1A\). In contrast, \(\beta 1A\) contains a novel juxtamembrane region that is 55 amino acids larger than that found in \(\beta 1\). This structural difference may be responsible for the opposite effects on steady state inactivation by \(\beta 1\) versus \(\beta 1A\).
FIG. 7. Effects of β1A on the functional properties of whole cell sodium currents. A, voltage-dependent sodium currents recorded in a SNaIIA cell (top traces) and a SNaIIAβ1A cell (bottom traces). Currents were elicited by depolarizations to −40, −30, −20, −10, 0 and +10 mV, from a prepulse potential of −100 mV. B, mean activation (filled symbols) and inactivation (open symbols) curves for cell lines SNaIIA (circles), SNaIIAβ1A-7 (squares), SNaIIAβ1A-8 (triangles), and SNaIIAβ1A-16 (inverted triangles). For each cell, activation and inactivation were analyzed as described under “Experimental Procedures.” The symbols show means of the activation and inactivation data for the different cell lines. In this figure and Fig. 8, error bars indicate S.E. The smooth lines were generated with the Boltzman equation (see “Experimental Procedures”), using mean values of V1/2 and k determined for each cell line from fits of individual experiments. Mean values for V and k and the number of experiments for each cell line are as follows: activation, SNaIIA, V = −11.0 ± 0.96, k = −8.3 ± 0.28, n = 15; SNaIIAβ1A-7, V = −8.1 ± 2.13, k = −7.9 ± 0.48, n = 6; SNaIIAβ1A-8, V = −11.6 ± 1.21, k = −7.4 ± 0.31, n = 11; SNaIIAβ1A-16, V = −17.0 ± 1.41, k = −7.18 ± 0.39, n = 11; inactivation: SNaIIA, V = −48.3 ± 0.77, k = 6.5 ± 0.20, n = 13; SNaIIAβ1A-7, V = −45.2 ± 1.25, k = 6.8 ± 0.22, n = 6, SNaIIAβ1A-8, V = −45.9 ± 0.63, k = 6.5 ± 0.21, n = 11; SNaIIAβ1A-16, V = −47.2 ± 0.50, k = 6.5 ± 0.15, n = 10. C, inactivation time constants (τ inactivation) determined from fits of current decay for SNaIIA (○), SNaIIAβ1A-7 (□), SNaIIAβ1A-8 (△), SNaIIAβ1A-16 (◆) and SNaIIAβ1 (○) cells, plotted as a function of test potential. D, mean V1/2 values for activation (filled symbols) and inactivation (open symbols) for cell lines SNaIIA, SNaIIAβ1A-7, SNaIIAβ1A-8, SNaIIAβ1A-16, and SNaIIAβ1.

In addition to opposite effects on steady state inactivation, the voltage dependence of activation and the rate of channel inactivation were also different in one of the three β1A-expressing cell lines, compared with the parent SNaIIA cell line. Thus, whole cell electrophysiological data suggest that β1A subunits may subtly modulate various aspects of sodium channel function. Nevertheless, the differences between cell lines with and without β1A subunits in the properties of whole cell sodium currents were very small and/or not observed in all cell lines. Therefore, additional analysis, perhaps at the single channel level, will be necessary to resolve whether these small differences actually reflect modulation of sodium channel function by β1A or some other source of variability between cell lines.

TTX-sensitive sodium channel α subunits expressed in brain (SCN1A, Ref. 39; SCN2A, Refs. 4 and 10; SCN3A, Ref. 5; SCN8A, Ref. 40) and skeletal muscle (SCN4A, Ref. 9) have been shown to be modulated by coexpression of β1 subunits in heterologous systems. In contrast, TTX-resistant sodium channel α subunits expressed in cardiac myocytes (SCN5A, Ref. 41) and peripheral nerve (SNS/PN3/SCN10A, Refs. 42–44) are much less sensitive or insensitive to modulation by β1 when co-expressed either in Xenopus oocytes or mammalian cells. A transcript encoding a predicted TTX-insensitive sodium chan-

FIG. 8. Effect of β1A on the level of expression of functional sodium channels. A, current densities for SNaIIA, SNaIIAβ1A, and SNaIIAβ1 cell lines. Currents were elicited by depolarization to +10 mV from a prepulse potential of −100 mV. Peak current amplitude was divided by cell capacitance to give current density. Cell capacitance was determined by integrating the area under transients elicited by 3-mV voltage steps applied before series resistance compensation and capacitive transient cancellation. Mean capacitance measurements for the four different cell lines were not significantly different, indicating that coexpression of β1A or β1 did not alter cell surface area. B, amplitude-frequency histogram for SNaIIA (black bars) and SNaIIAβ1 (white bars; data for all three SNaIIAβ1A cell lines were combined). Currents were evoked by depolarization to +10 mV. The bars indicate the number of cells with peak currents that fell within different amplitude ranges.

Acknowledgments—We thank Patrick Oh (University of Michigan) and Hongling Li (McGill University) for excellent technical assistance.

2 J. D. Malhotra and L. L. Isom, unpublished results.
3 J. D. Malhotra, M. Hortsch, and L. L. Isom, unpublished results.
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