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Comparison of Fura-2 Imaging and Electrophysiological Analysis of Murine Calcium Channel α1 Subunits Coexpressed with Novel β2 Subunit Isoforms

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SUMMARY

A polymerase chain reaction product was used to isolate mouse brain cDNA clones coding for isoforms of the β subunit of voltage-dependent Ca^{2+} channels. The two mouse brain β2 subunit cDNA clones described, β2a and β2b, differed by alternative splicing within the coding region but possessed a unique amino terminus not yet reported in other β2 subunit cDNAs. Northern blot and RNase protection analyses demonstrated that both mRNA isoforms could be detected in highest abundance in heart and brain and at lower levels in lung, kidney, and testis. In a novel assay for β2 subunit function, COS-1 cells were transfected with α1 and β2 subunit expression vectors and assayed for increases in intracellular Ca^{2+} concentration by using fura-2 imaging. Co-transfection of COS-1 cells with the mouse brain class C-I α1 subunit expression vector and either of the β2 subunit expression vectors resulted in increases in intracellular Ca^{2+} concentration after stimulation with elevated K^+ and the dihydropyridine agonist Bay K 8644. Transfection of either α1 or β2 subunit expression vectors alone did not result in an elevation of intracellular Ca^{2+} concentration. Electrophysiological recording of human embryonic kidney 293 cells transfected with the expression vector for the α1 subunit alone or with either β2 subunit demonstrated expression of voltage-dependent Ca^{2+} channels that were dihydropyridine sensitive. Currents formed by expression of only the α1 subunit were small and slowly inactivated. In contrast, the currents formed by coexpression of α1 subunits with either β2 subunit were larger and inactivated more rapidly. Dihydropyridine binding studies demonstrated that coexpression of α1 subunits with β2 subunits increased the density of functional receptors, compared with expression of α1 subunits alone. These experiments suggested that coexpression of the α1 and β2 subunits produced functional dihydropyridine-sensitive Ca^{2+} channels and that both β subunit isoforms had modulatory effects on these channels.

The predominant pathway by which Ca^{2+} enters neurons, endocrine cells, and muscle cells is through voltage-dependent Ca^{2+} channels present in the plasma membrane (1–3). Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels regulates a number of cellular functions, such as muscle contraction (4), neurotransmitter release (5), and gene expression (6). Based on their electrophysiological and pharmacological properties, these voltage-dependent Ca^{2+} channels have been classified into multiple types, including L, N, T, and P (2). The L-type channel is a high-threshold, slowly inactivating channel that is modulated by dihydropyridine agonists and antagonists. The N-type channel is a high-threshold, rapidly inactivating channel that is blocked by the *Conus geographus* peptide toxin ω-conotoxin GVIA. The P-type channel is a high-threshold channel that is blocked by the peptide toxin ω-agatoxin IVa, isolated from the funnel web spider *Agelenopsis aperta*. In contrast to these channels, the T-type channel is a low-threshold channel that is preferentially blocked by Ni^{2+}.

Skeletal muscle contains the highest concentrations of voltage-sensitive Ca^{2+} channels that are of the L type (4). Purification of L-type channels from skeletal muscle led to the isolation of a protein complex composed of five subunits, α1, β, γ, α2, and δ (7). Molecular cloning of these different subunits has demonstrated molecular heterogeneity for α1 and β subunits. In contrast, α2, δ, and γ subunits do not exhibit such diverse molecular heterogeneity.

The α1 subunit is the largest of the five subunits and is an integral membrane protein. It also forms the ion-selective

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ABBREVIATIONS: PCR, polymerase chain reaction; kb, kilobase(s); $[Ca^{2+}]_i$, intracellular calcium concentration; CMV, cytomegalovirus; PBS, phosphate-buffered saline; HEK, human embryonic kidney; bp, base pair(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pore through which Ca^{2+} flows into the cell (8). Molecular cloning of $\alpha 1$ subunits from rat brain suggests the existence of at least five different genes, each with several splice variants (9–13). The five classes of rat brain $\alpha 1$ subunits have been designated A, B, C, D, and E (9, 12). The class A $\alpha 1$ subunit gene may code for the P-type channel $\alpha 1$ subunit, but recent work demonstrated significant differences between recombinant currents and native P-type currents from neuronal cells (14). The class B $\alpha 1$ subunit gene has been shown to code for the N-type channel (13). The class C $\alpha 1$ subunit gene codes for cardiac L-type $\alpha 1$ subunits that are also expressed in smooth muscle and brain (15). The class D $\alpha 1$ subunit gene codes for a neuroendocrine L-type $\alpha 1$ subunit (16). The class E $\alpha 1$ subunit gene codes for an $\alpha 1$ subunit that forms a high-threshold current that is insensitive to dihydropyridines and ω-agatoxin IVA but is inhibited by Ni^{2+} (12, 17).

The $\alpha 2$, δ, and γ subunits are membrane-associated proteins (18–20). The $\alpha 2$ and δ subunits are derived by proteolysis of a precursor protein and are associated by disulfide bonds. To date, only one $\alpha 2$ isoform has been cloned. It was initially cloned from skeletal muscle and subsequently shown to be expressed in brain and heart as alternate splice variants (16). The γ subunit is a membrane-associated protein that was originally cloned from skeletal muscle (20). Attempts to clone this subunit from other tissues have been unsuccessful, suggesting that it is not a component of Ca^{2+} channels expressed in heart or brain (20). In addition, purification from brain demonstrated that neuronal L-type Ca^{2+} channels are composed of only $\alpha 1$, $\alpha 2\delta$, and β subunits (21).

In contrast to the $\alpha 1$, $\alpha 2$, and γ subunits, the β subunit is a cytoplasmic protein that is believed to associate tightly with the cytoplasmic domain of the $\alpha 1$ subunit (22). Molecular biological studies of β subunits suggest the existence of at least four different genes and several splice variants. The four classes of β subunits are designated as $\beta 1$–$\beta 4$. $\beta 1$ is expressed in skeletal muscle and in brain as a splice variant (23, 24). $\beta 2$ is localized to cardiac and neuronal tissue (25). $\beta 3$ is found predominantly in brain and forms a component of N-type Ca^{2+} channels (26, 27). $\beta 4$ is expressed at high levels in cerebellum (28). Coexpression of cardiac and skeletal muscle L-type $\alpha 1$ subunits with various β subunit cDNAs demonstrated that the β subunit plays a major role in modulation and expression of functional Ca^{2+} channels. Expression of various β subunits with $\alpha 1$ subunits resulted in increased peak currents, altered rates of activation and inactivation, and increased density of functional channels (29–31).

The structural composition of neuronal Ca^{2+} channels is not well understood, due to their lower abundance and molecular heterogeneity. Recently we reported the cloning and expression of a mouse brain class C-I $\alpha 1$ subunit clone, which was able to form functional Ca^{2+} channels in cells that contain endogenous β subunit proteins (32). Here we report the cloning of two novel cDNA clones (mouse brain $\beta 2$a and $\beta 2$b) that encode alternative splice variants of the $\beta 2$ gene. These cDNA clones were used to demonstrate that transient expression of either $\beta 2$ splice variant with the previously described murine calcium channel $\alpha 1$ subunit clone (mbC-I) can form functional dihydropyridine-sensitive Ca^{2+} channels. These channels were detected and characterized by fura-2 imaging, electrophysiological recordings, and dihydropyridine binding. In addition, this work examines differential effects of alternatively spliced $\beta 2$ subunits, which differ only in internal amino acid sequence. This work also characterizes the class C-I $\alpha 1$ subunit, which had not been previously examined using electrophysiological recordings.

Materials and Methods

Amplification of a rabbit skeletal muscle β subunit cDNA fragment from total RNA. Two oligonucleotide primers (GGTCTAGACGGAGGAGAGGCTCCCCTCCAT and CCGAATTCGTCAAACAAAGCTTTCTGCATCAT) were used to amplify a rabbit skeletal muscle β subunit cDNA fragment from rabbit skeletal muscle first-strand cDNA. The amplified product codes for a fragment containing sequence from nucleotide 136 to nucleotide 1052, based on the published rabbit skeletal muscle β subunit cDNA sequence (23). A PCR product of 916 bp was isolated on a 1% agarose gel. The isolated fragment was then digested with *Eco*RI and *Xba*I and ligated into *Eco*RI/*Bgl*II-digested pGEM-3Zf(+). After base denaturation, the resulting plasmids were sequenced with SP6 and T7 promoter primers, using a modified T7 DNA polymerase (Sequenase).

Mouse brain β subunit cDNA cloning and sequencing. Approximately 1.0×10^6 recombinant λZapII phage containing mouse brain cDNA fragments (Stratagene) were screened by hybridization as described previously (33). The 916-bp *Eco*RI/*Xba*I rabbit skeletal muscle β subunit cDNA fragment was labeled by random primer extension in the presence of [α-^{32}P]dATP. The resulting radiolabeled DNA fragments were hybridized to phage DNA immobilized on Hybond-N membranes. Initially, six cDNA clones were isolated and sequenced with T3 and T7 promoter primers, using modified T7 DNA polymerase (Sequenase). One clone (mbβ1) encoded a splice variant of the mouse skeletal muscle β subunit that contained an open reading frame but lacked an initiator methionine. The 1.5-kb *Eco*RI/*Xho*I fragment from mbβ1 was isolated and labeled by random primer extension in the presence of [α-^{32}P]dATP. The library was rescreened, resulting in the isolation of 54 additional clones. The cDNA inserts were sequenced with T3 and T7 promoter primers. Two clones, $\beta 2$a and $\beta 2$b, were sequenced entirely. Sequence alignment of the amino acid sequences was performed using the Genetics Computer Group sequence analysis programs and reiterative alignment of the sequences using the GAP program. The sequences were submitted to the GenBank/EMBL Data Bank with accession number L20343.

Northern blot and RNase protection analyses. A 460-bp *Eco*RI/*Sph*I cDNA fragment was isolated from $\beta 2$a, subcloned into *Eco*RI/*Sph*I-digested pGEM-4, and sequenced. Plasmid was linearized with *Eco*RI and used as a template to generate antisense RNA probes for both β subunits. cRNA probes containing [α-^{32}P]UTP (ICN Biochemicals) were generated using T7 RNA polymerase (BRL). RNase protection analysis using the 460-bp *Eco*RI/*Sph*I fragment was performed according to previously described methods (34). Expression of $\beta 2$a was detected by an asymmetric PCR-generated probe, encoding a 131-bp insert not found in $\beta 2$b. Two oligonucleotide primers (ATAGACATAGATGCTAC and CTTCTTAAAGAAGGGCA) were used to amplify a 131-bp fragment from the $\beta 2$a cDNA template. The probe was amplified from the 131-bp fragment by asymmetric PCR using the second 3′ oligonucleotide primer described above. The RNA blot (Clonetech) contained 2 μg of poly(A)$^+$ RNA from the various tissues indicated and was the gift of Drs. Karen Martell and Jack Dixon (University of Michigan, Department of Biochemistry). After prehybridization for 4 hr, Northern blot hybridization with 1×10^6 cpm/ml cRNA or asymmetric PCR probe was performed at 60° overnight. The blot was washed at room temperature for 15 min in 2× SSPE (1× SSPE is 0.15 M NaCl, 5 mM NaH_2PO_4, 1 mM EDTA) with 0.1% sodium pyrophosphate and 0.5% SDS, followed by washes at 37°, 50°, and 70°. Blots were subjected to autoradiography at −80°.

Construction of CMV.$\beta 2$a and CMV.$\beta 2$b expression vectors. A 2.1-kb or 1.97-kb *Pst*I/*Pst*I fragment was isolated from $\beta 2$a or $\beta 2$b,

respectively. Each fragment was then ligated into the *Pst*I site of pBSBgl, a gift of Dr. Timothy Angelotti (University of Michigan, Department of Pharmacology). The pBSBgl plasmid was derived from pBluescript SK(−) that was digested with *Eco*RI and *Xho*I, end filled, and ligated with *Bgl*II linkers. The resultant plasmids were sequenced, and those containing an insert were digested with *Bam*HI and *Bgl*II. The cDNA fragments were isolated and ligated into the *Bgl*II site of pCMV.Neo (35). The resulting plasmids with the mbβ2a or mbβ2b coding region in the sense orientation were restriction mapped, sequenced to confirm orientation, and denoted pCMV.mbβ2a and pCMV.mbβ2b, respectively.

Transfection of COS-1 cells and fura-2 imaging. Two 10-cm plates containing COS-1 cells at 30% confluency were transfected, by calcium phosphate precipitation, with 10 μg/plate of pCMV.mbβ2a or pCMV.mbβ2b, 10 μg of pCMV.MCI (32), and 5 μg of pCMV.βGAL, as described previously (36). COS-1 cells were also transfected with either pCMV.Neo, pCMV.mbβ2a, pCMV.mbβ2b, or pCMV.MCI alone. Thirty-six hours after application of the DNA precipitate, the cells were treated with trypsin, resuspended in 5 ml of buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, 30 mM HEPES, pH 7.4), and centrifuged for 5 min. The cells were resuspended in 5 ml of buffer A and incubated with 2 μM fura-2/acetoxymethyl ester at room temperature for 30 min. The cells were then centrifuged and resuspended in fresh buffer A. For measurement of $[Ca^{2+}]_i$, fura-2-loaded cells were transferred to a closed chamber mounted on the stage of a Zeiss Axiovert inverted microscope and were continuously superfused at 1 ml/min with buffer A, at 37°. Solution changes were rapidly accomplished by means of a valve attached to an eight-chambered superfusion reservoir. Activation and blockade of calcium channels were elicited by superfusion with 55.6 mM KCl and 1 μM Bay K 8644 and were inhibited by 100 μM nifedipine. Measurement of $[Ca^{2+}]_i$ was performed using a digital imaging system (Attofluor, Rockville, MD). Excitation of cells at 334 and 380 (10-nm bandpass) was accomplished with a computer-selectable filter and shutter system. Resultant emission at 510 nm was monitored with an intensified charge-coupled device camera and subsequently digitized. Calibration of $[Ca^{2+}]_i$ signals was performed by applying the equation of Grynkiewicz *et al.* (37). The fluorescent emission of external Ca^{2+} standards containing 1 μM fura-2 either in the absence (1 mM EGTA) or in the presence of Ca^{2+} (1 mM) was monitored, allowing the determination of R_{max}, R_{min}, and β. The calibration was then applied to user-defined areas of interest, where gray level values were computed to give a measurement of $[Ca^{2+}]_i$, as described previously (38).

Transfection of HEK 293 cells and electrophysiological recording. Six-centimeter plates containing HEK 293 cells at 75% confluency were transfected, by calcium phosphate precipitation, with 10 μg/plate of pCMV.β2a or pCMV.β2b, 10 μg of pCMV.MCI, and 1 μg of pCMV.βGAL, as described previously (39). Twenty-four hours later, the cells were treated with trypsin, resuspended in 2 ml of Dulbecco's modified Eagle's medium, and incubated with 75 μl of 10 mg/ml DNase I for 5 min. The cells were centrifuged and plated onto Mecanex-gridded dishes at a density of 100,000 cells/dish. Forty-eight hours after transfection, a β-galactosidase staining protocol was used to identify transfected cells (39). Cells were maintained in medium at 37°, rinsed with Dulbecco's phosphate buffered saline (PBS) (with Ca^{2+} and Mg^{2+}), and returned to 37°. During incubation of the cells, 1 mM fluorescein di-β-galactopyranoside (a fluorescent *lacZ* substrate) in 0.5× PBS was warmed to 37°. Cells were removed from incubation, the PBS was aspirated, fluorescein di-β-galactopyranoside solution was added to the cells, and the culture dish was warmed at 37° for 1 min. The dish was then placed on ice and cold PBS was added. After 5 min, cells were viewed by fluorescence microscopy, to localize cells expressing β-galactosidase. After scoring of fluorescein-positive cells, the culture dish was removed for electrophysiological recordings.

Whole-cell voltage-clamp recordings of Ca^{2+} currents were made by the whole-cell variation of the patch-clamp technique. Recordings were made using glass micropipettes (Fisher microhematocrit capillary tubes; inner diameter, 1.1 mm) pulled in four stages on a Brown-Flaming micropipette puller (model P-80) to 3–8 MΩ. Micropipettes were filled with a solution of 140 mM CsCl, 30 mM CsOH, 10 mM HEPES, 10 mM EGTA, 5 mM ATP, and 0.1 mM GTP, pH 7.3 (290 mOsM). Cells were bathed in a solution of 13 mM $BaCl_2$, 67 mM choline chloride, 100 mM tetraethylammonium chloride, 5.3 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.40 (330 mOsM). All experiments were conducted at room temperature.

Signals were led to an Axopatch 1-B patch-clamp amplifier (Axon Instruments, Burlingame, CA), which corrected pipette and whole-cell capacitance and series resistance by compensation circuitry. Typical input resistances were 500 MΩ to 1 GΩ. Series resistance was estimated by cancellation of the capacitance charging current transient after patch rupture. Series resistance compensation was 80–100% in most cases. After patch rupture, the cell was hyperpolarized to a holding potential of −80 mV. Voltage step commands were generated, digitized at 5.6 kHz, stored, and analyzed by a microcomputer (IBM AT) using the program pClamp 5.5.1 (Axon Instruments). Current traces were filtered with a Bessel filter at 10 kHz (−3 dB). Holding current was subtracted to base-line and leak currents were estimated using hyperpolarizing voltage commands of the same magnitude as the depolarizing commands used to evoke inward currents. The resulting current was assumed to be equal and opposite in magnitude to the leak current and was digitally subtracted from the recorded inward current to obtain the total Ba^{2+} current. Estimates of current magnitude and kinetic parameters were expressed as mean ± standard error throughout.

Drugs were freshly prepared or taken from stock solutions on the day of the experiments, dissolved in external solution, and drawn up into blunt-tipped micropipettes (40–50 μm) that were positioned about 50 μm from the cell. Drugs were usually applied by passive diffusion before and during voltage step commands. Currents were evoked during delivery of the drug to attain a constant drug concentration at the cell. Micropipettes containing different drugs were substituted sequentially, with control and recovery trials, and were removed from the bath solution when not in use. Application of diluent alone had no effect on evoked currents.

(+)-[^{3}H]PN200–110 binding to transfected HEK 293 cells. Ten-centimeter plates containing HEK 293 cells at 75% confluency were transfected, by calcium phosphate precipitation, with 10 μg/plate of pCMV.β2a or pCMV.β2b, 10 μg of pCMV.MCI, and 2 μg of pCMV.βGAL, as described previously (39). Binding was determined as described previously (40), with the following modifications. Transiently transfected HEK 293 cells were harvested in 50 mM Tris·HCl, pH 7.4, homogenized in a homogenizer using a Teflon pestle, and centrifuged at 20,000 × *g* for 20 min. Supernatants were assayed for β-galactosidase activity as described previously, to normalize transfection efficiency (35). Membrane pellets were resuspended in 50 mM Tris·HCl. Binding was assayed by incubation of 1 mg of membrane protein with 1 nM (+)-[^{3}H]PN200–110. Nonspecific binding and total binding were determined by the addition of 1 μM nifedipine or buffer, respectively. Binding reactions were incubated for 2 hr at 25° and were terminated by filtration through Whatman GF/C glass fiber filters, under vacuum, and washing with cold binding buffer. Bound radioligand was measured by liquid scintillation counting.

Results

Isolation of mouse brain cDNA clones for calcium channel β2 subunits. A 916-bp cDNA fragment of the rabbit skeletal muscle β subunit was used to screen a mouse brain λZapII cDNA library. Evidence suggested that the skeletal muscle β subunit was expressed in brain; thus, a skeletal muscle β subunit was presumed to have a high probability of hybridizing to neuronal isoforms (24). The mouse brain β1 cDNA, which was isolated first, encoded an incomplete splice

variant of the skeletal muscle β1 subunit. A 1.5-kb *Eco*RI/*Xba*I fragment of β1 was used to rescreen the library under low stringency conditions. Fifty-four additional cDNA clones were isolated by rescreening the library. Upon sequencing, two cDNA clones (mouse brain β2a and β2b) were shown to encode splice variants homologous to the β2 subunit described previously (25). The individual cDNA sequences were aligned to generate the composite cDNA and predicted amino acid sequences shown in Fig. 1. Both β2a and β2b have amino termini that differ significantly from the previously described rat β2 sequence, up to amino acid 17. To date, an homologous amino-terminal sequence has not been described. The β2a cDNA contained 132 nucleotides, coding for amino acids 177–220, that were not present in mbβ2b. In contrast, β2b contained 18 nucleotides, which deleted amino acids 177–220 and replaced them with six amino acids. Putative cAMP-dependent protein kinase (Thr-171) and protein kinase C (Thr-30 and Ser-78, -149, -166, -197, -232, -242, -352, -496, and -594) phosphorylation sites present in the β subunit isoforms are shown in Fig. 1.

Northern blot and RNase protection analysis of β subunit expression. To determine the relative expression of both β2a and β2b mRNA, Northern blot and RNase protection analyses were performed. Hybridization with an antisense β2a ^{32}P-labeled riboprobe demonstrated that β2-related mRNA was detected in heart, brain, lung, kidney, and testis. In these tissues three mRNAs, of approximately 6.0, 4.0, and 3.5 kb, were detected, as seen in Fig. 2A. As suggested for the rat β2 mRNA (25), the three mRNA species may be due to alternative polyadenylation. The β2 subunit mRNA was not detected in spleen, liver, or skeletal muscle. Hybridization with an antisense asymmetric PCR probe encoding a 132-bp insert found only in β2a detected mRNA in heart, brain, and lung, as seen in Fig. 2B. However, as shown in Fig. 2C, RNase protection analysis with antisense β2a detected expression of both isoforms in heart, brain, lung,

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CTCCGTGGATCTGTGCCCTGGGTGCAGCTGCGGAAGATAAAGGCGCTACCCGGCTCATGAAGGCCACCTGGATCAGGCTTCTGAAAAGAGCCAAGGGAGGAAGGCTGAAGAGTTCGGACA    120
                                                          M  K  A  T  W  I  R  L  L  K  R  A  K  G  G  R  L  K  S  S  D  I  22

TCTGTGGTTCGGCAGACTCCTACACCAGCCGCCCATCCGATTCAGATGTGTCTTTGGAAGAGGACCGGGAGGCAGTACGTAGAGAAGCTGAGCGGCAGGCACAGGCACAGTTGGAAAAAG    240
  C  G  S  A  D  S  Y  T  S  R  P  S  D  S  D  V  S  L  E  E  D  R  E  A  V  R  R  E  A  E  R  Q  A  Q  A  Q  L  E  K  A  62

CAAAGACAAAACCTGTTGCATTTGCAGTTCGGACCAATGTCAGATACAGCGCACGCCAGGAGGATGACGTTCCGGTGCCTGGCATGGCCATCTCCTTCGAGGCCAAAGATTTTCTGCATG    360
  K  T  K  P  V  A  F  A  V  R  T  N  V  R  Y  S  A  R  Q  E  D  D  V  P  V  P  G  M  A  I  S  F  E  A  K  D  F  L  H  V  102

TTAAAGAAAAATTTAATAATGACTGGTGGATAGGACGGCTGGTTAAAGAAGGCTGTGAAATCGGATTTATTCCAAGCCCAGTCAAACTAGAGAACATGAGGCTACAGCACGAACAGAGAG    480
  K  E  K  F  N  N  D  W  W  I  G  R  L  V  K  E  G  C  E  I  G  F  I  P  S  P  V  K  L  E  N  M  R  L  Q  H  E  Q  R  A  142

CCAAGCAAGGGAAATTCTATTCCAGTAAATCAGGAGGGAATTCATCATCCAGTTTGGGTGACATAGTGCCCAGTTCCAGAAAATCGACACCGCCGTCATCTGCTATAGACATAGATGCTA    600
  K  Q  G  K  F  Y  S  S  K  S  G  G  N  S  S  S  S  L  G  D  I  V  P  S  S  R  K  S  T  P  P  S  S  A  I  D  I  D  A  T  182
                                                                                                                  [.............
                                                                                                                  [.............

CTGGCTTAGATGCAGAAGAGAATGATATTCCAGCAAACCACCGCTCCCCTAAGCCCAGTGCAAACAGTGTAACATCACCCCACTCCAAAGAGAAAAGAATGCCCTTCTTTAAGAAGACAG    720
  G  L  D  A  E  E  N  D  I  P  A  N  H  R  S  P  K  P  S  A  N  S  V  T  S  P  H  S  K  E  K  R  M  P  F  F  K  K  T  E  222
.....................................................................................AAGCAGAAGCAGAAGTCG]
.....................................................................................K  Q  K  Q  K  S  ]

AGCACACTCCTCCTTATGATGTGGTACCTTCCATGCGACCAGTGGTGCTGGTGGGCCCGTCTCTGAAGGGGTATGAGGTCACAGATATGATGCAAAAAGCACTGTTTGATTTTCTAAAAC    840
  H  T  P  P  Y  D  V  V  P  S  M  R  P  V  V  L  V  G  P  S  L  K  G  Y  E  V  T  D  M  M  Q  K  A  L  F  D  F  L  K  H  262

ACAGATTTGAAGGGCGGATATCCATCACAAGAGTCACTGCTGACATCTCCCTTGCCAAACGCTCAGTATTAAACAATCCCAGTAAGCATGCAATCATAGAAAGATCCAACACAAGGTCCA    960
  R  F  E  G  R  I  S  I  T  R  V  T  A  D  I  S  L  A  K  R  S  V  L  N  N  P  S  K  H  A  I  I  E  R  S  N  T  R  S  S  302

GCTTAGCGGAAGTTCAGAGTGAAATTGAAAGGATTTTTGAACTTGCAAGAACACTGCAATTGGTAGTCCTTGACGCGGATACAATTAATCACCCAGCTCAACTCAGTAAGACCTCTTTGG    1080
  L  A  E  V  Q  S  E  I  E  R  I  F  E  L  A  R  T  L  Q  L  V  V  L  D  A  D  T  I  N  H  P  A  Q  L  S  K  T  S  L  A  342

CCCCTATCATAGTGTACGTAAAGATTTCTTCTCCCAAGGTTTTACAAAGGTTGATAAAATCTCGAGGGAAATCTCAAGCAAAACACCTCAATGTCCAGATGGTAGCAGCTGATAAACTGG    1200
  P  I  I  V  Y  V  K  I  S  S  P  K  V  L  Q  R  L  I  K  S  R  G  K  S  Q  A  K  H  L  N  V  Q  M  V  A  A  D  K  L  A  382

CCCAATGTCCTCCGCAGGAATCATTTGATGTGATCTTGGATGAGAACCAGCTGGAGGACGCTTGTGAGCATCTTGCCGACTATCTGGAGGCATACTGGAAGGCCACCCACCCTCCCAGCG    1320
  Q  C  P  P  Q  E  S  F  D  V  I  L  D  E  N  Q  L  E  D  A  C  E  H  L  A  D  Y  L  E  A  Y  W  K  A  T  H  P  P  S  G  422

GTAACCTCCCCAACCCTCTGCTTAGCCGGACTTTAGCCTCCTCAACTTTACCTCTGAGCCCCACCCTCGCCTCTAATTCACAGGGTTCTCAAGGTGATCAAAGGCCTGATCGCTCTGCTC    1440
  N  L  P  N  P  L  L  S  R  T  L  A  S  S  T  L  P  L  S  P  T  L  A  S  N  S  Q  G  S  Q  G  D  Q  R  P  D  R  S  A  P  462

CCCGGTCTGCTTCCCAAGCTGAAGAAGAACCTTGCCTAGAACCCGTCAAAAAATCCCAACACCGTTCCTCCTCAGCCACACACCAAAACCACCGCAGCGGGACAGGTCGAGGCCTCTCTA    1560
  R  S  A  S  Q  A  E  E  E  P  C  L  E  P  V  K  K  S  Q  H  R  S  S  S  A  T  H  Q  N  H  R  S  G  T  G  R  G  L  S  R  502

GGCAAGAGACGTTTGACTCTGAAACCCAAGAGAGCCGAGACTCTGCCTACGTGGAGCCAAAGGAAGATTATTCACATGAACATGTGGACCGGTATGTCCCACACCGCGAGCATAACCACA    1680
  Q  E  T  F  D  S  E  T  Q  E  S  R  D  S  A  Y  V  E  P  K  E  D  Y  S  H  E  H  V  D  R  Y  V  P  H  R  E  H  N  H  R  542

GAGAGGAGACCCACAGCAGCAATGGCCACAGACACAGGGAGTCTCGCCACCGCTCTAGGGACATGGGTCGAGACCAGGACCACAATGAGTGCATCAAACAACGAAGCCGGCATAAATCTA    1800
  E  E  T  H  S  S  N  G  H  R  H  R  E  S  R  H  R  S  R  D  M  G  R  D  Q  D  H  N  E  C  I  K  Q  R  S  R  H  K  S  K  582

AGGATCGCTACTGTGACAAGGAGGGGGAAGTAATATCCAAGAGAAGGAATGAGGCTGGCGAGTGGAACAGGGATGTATACATCCGCCAATGACCGTGCGTGTCCCCTACCCCCAAGTCTT    1920
  D  R  Y  C  D  K  E  G  E  V  I  S  K  R  R  N  E  A  G  E  W  N  R  D  V  Y  I  R  Q                                    611

TGTGTATCATCCTTAAGCAAAATCTTTGGGGGTTACATTGCAACCA  1966
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Fig. 1. Consensus sequence of mouse brain β2 subunit cDNA clones. The consensus nucleotide sequence derived from the two independent cDNA clones is shown, with the predicted amino acid sequence below the nucleotide sequence. The region where alternatively spliced forms of cDNA clones were detected is indicated by the second nucleotide and amino acid sequences (in *brackets*) (nucleotides 585–716). Potential phosphorylation sites for cAMP-dependent protein kinase (Thr-171) and protein kinase C (Thr-30 and Ser-78, -149, -166, -167, -197, -232, -242, -352, -496, and -594) are *underlined*.

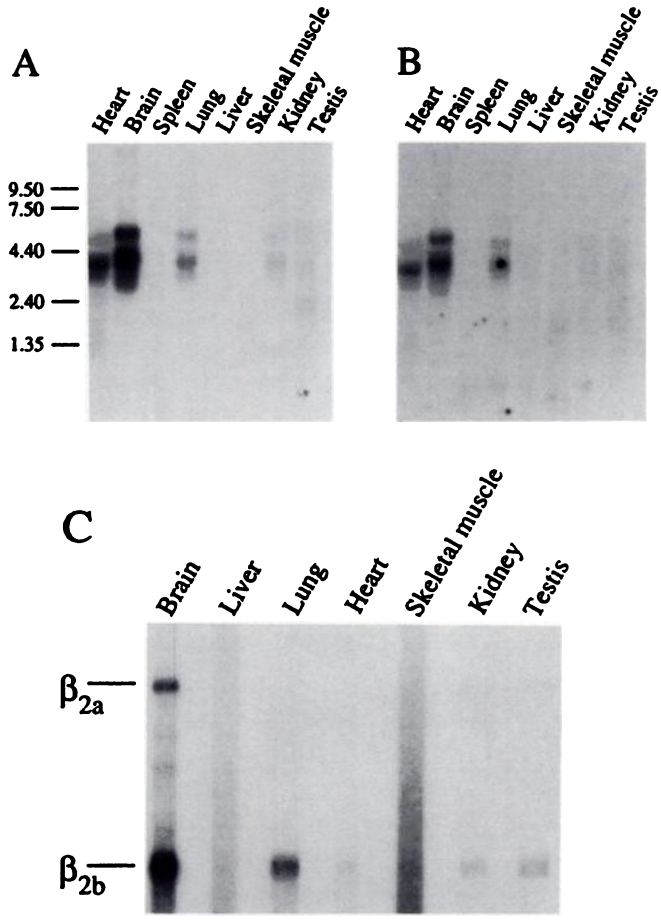

Fig. 2. Tissue distribution of β2 subunit mRNA. RNA from various tissues was analyzed by Northern blot and RNase protection analyses as described in Materials and Methods. *Numbers to the left*, sizes of the RNA standards (in kb). A, Northern blot analysis of β2 subunit mRNA expression in various tissues, determined by hybridization with an antisense riboprobe transcribed from a 460-bp *Eco*RI/*Sph*I β2a fragment. B, Northern blot analysis of β2a subunit mRNA expression, determined by hybridization with an asymmetric PCR probe encoding a 126-bp insert found only in mbβ2a. C, RNase protection analysis of β2a and β2b subunit mRNA expression in tissues, determined by hybridization with the 460-bp *Eco*RI/*Sph*I β2a fragment described for A.

kidney, and testis. This result was most likely due to the greater sensitivity of RNase protection assays and the low level of β2 mRNA in kidney and testis.

Fura-2 imaging of transiently transfected COS-1 cells. To determine the functional expression of Ca^{2+} channels, COS-1 cells were transfected with both mouse brain class C-I α1 subunit and β2 subunits, and fura-2 imaging was then performed to detect increases in $[Ca^{2+}]_i$ due to the influx of Ca^{2+} after depolarization-induced opening of Ca^{2+} channels. Increases in $[Ca^{2+}]_i$ in cells co-transfected with either β2 subunit expression vector and the α1 subunit expression vector were measured after superfusion with the Ca^{2+} channel agonist Bay K 8644 and 55.6 mM KCl. Fura-2 imaging estimated an increase in $[Ca^{2+}]_i$ in individual cells from a large population, but only cells transfected with α1 and β2 cDNAs responded to Bay K 8644 and KCl. Bay K 8644 and KCl did not stimulate an increase in $[Ca^{2+}]_i$ in mock-transfected cells or in cells transfected with either α1 or β2 subunit cDNAs alone (Fig. 3, A–D). In contrast, $[Ca^{2+}]_i$ was increased in cells coexpressing β2a or β2b and α1 subunits by as much as 250 nM (Fig. 3, E–H). Typically, 20–25% of imaged cells showed increased $[Ca^{2+}]_i$ upon perfusion with Bay K 8644 and KCl (Fig. 4B). The number of cells responsive to Bay K 8644 and KCl correlated well with the transfection efficiency, as determined by β-galactosidase staining (data not shown). Calcium channel activity was not observed during superfusion with Bay K 8644, KCl, and 100 μM nifedipine (Fig. 3, E and F).

Electrophysiological recordings of transiently transfected HEK 293 cells. Electrophysiological recordings were made from 172 transiently transfected, fluorescein-positive, HEK 293 cells with α1, α1β2a, α1β2b, or β2b subunit combinations. Interestingly, cells transfected with α1 subunits alone had greater viability than did α1β2 subunit combinations under identical conditions of cell preparation and recording (data not shown). The percentages of fluorescein-positive cells from which currents could be evoked were as follows: α1, 51% (25 of 49 cells); α1β2a, 40% (25 of 62 cells); α1β2b, 36% (17 of 47 cells); β2b, 0% (0 of 14 cells).

Currents evoked from cells transfected with α1 subunits alone were generally smaller than those from cells transfected with α1β2 subunit combinations. Bay K 8644 (10 μM) was required to detect α1 currents in 40% (10 of 25) of cells but was required in only 7% (3 of 42) of cells with α1β2 currents. The mean peak amplitude for cells expressing α1 subunit currents was 96.4 ± 27.2 pA (n = 25). The α1 subunit peak currents of >150 pA were enhanced by 67% (n = 3) upon application of Bay K 8644; α1 currents of <150 pA were undetected in the absence of Bay K 8644 (data not shown). Nifedipine (10 μM) blocked α1 currents by 80% within 2 min and by 100% with longer application times. There was no apparent voltage dependence of the current block by nifedipine (holding potentials of −80 to −40 mV and clamp potentials of −10 to 0 mV) (data not shown). Currents with peak amplitudes of <150 pA were difficult to assess and were not included in the analysis of kinetic parameters. The α1 subunits were activated to peak current rapidly, with an activation time of 20.8 ± 3.2 msec (n = 4). Representative current traces and a current-voltage relation for α1 currents are shown in Fig. 4A; they demonstrated that peak currents occurred at −10 mV and the reversal potential was 60 mV. Inactivation was slower for α1 subunit currents than for either α1β2 subunit current (Fig. 5A) and the time to 50% inactivation (half-decay time) for α1 currents was 793.3 ± 13.3 msec (n = 3) (Fig. 5B).

Fig. 4, B and C, *left*, shows current traces for α1β2a and α1β2b subunit combinations, respectively. Peak amplitudes for α1β2 subunit currents were 439.1 ± 109.3 pA (n = 25) (α1β2a) and 397.8 ± 74.6 pA (n = 16) (α1β2b). There was no significant difference between the mean peak current values for α1β2a and α1β2b currents (unpaired t test, p = 0.76). However, α1β2 currents were significantly larger than α1 currents (unpaired t test, p = 0.005 for α1β2a; p = 0.001 for α1β2b). The α1β2 control currents of >150 pA were enhanced by 62% (n = 15) upon addition of Bay K 8644 (10 μM). Bay K 8644 (10 μM) enhanced α1β2a and α1β2b currents in a manner similar to that described for α1 currents. Currents were activated fully by 23.2 ± 3.0 msec (n = 6) (α1β2a) and 22.0 ± 2.9 msec (n = 6) (α1β2b). There were no significant differences among the mean activation times to peak currents for α1, α1β2a, and α1β2b currents (analysis of variance, p = 0.87). Fig. 4, B and C, *right*, shows the current-voltage rela-

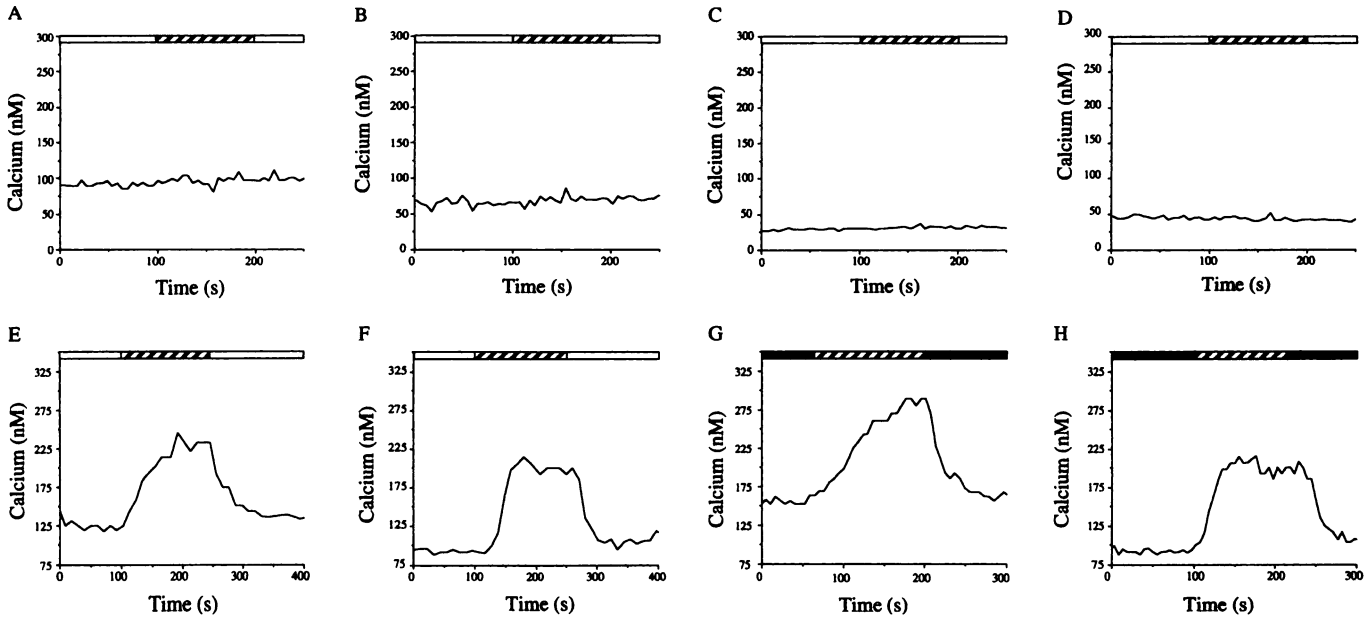

Fig. 3. Fura-2 imaging of transiently transfected COS-1 cells. Expression vectors were constructed as described in Materials and Methods. COS-1 cells were transfected, and 36 hr after transfection the cells were loaded with fura-2, superfused, and imaged as described in Materials and Methods. A, Transfection of COS-1 cells with pCMV.Neo. B, Transfection of COS-1 cells with pCMV.MC1. C, Transfection of COS-1 cells with pCMV.β2a. D, Transfection of COS-1 cells with pCMV.β2b. E and G, Co-transfection of COS-1 cells with pCMV.MC1and pCMV.β2a. F and H, Co-transfection of COS-1 cells with pCMV.MC1 and pCMV.β2b. □, Perfusion with buffer A; ▨, perfusion with 1 μM Bay K 8644 and 55.6 mM KCl; ■, perfusion with 1 μM Bay K 8644, 55.6 mM KCl, and 100 μM nifedipine. The expression experiments were repeated at least five times, and consistent results were obtained for each trial. The number of responsive cells correlated with the transfection efficiency, as determined by β-galactosidase staining.

tions for α1β2a and α1β2b subunits, respectively. Peak currents usually occurred at −10 mV (−20 mV for the α1β2b combination shown). Reversal potentials calculated for different current-voltage relations were variable (44 mV for α1β2a; 70 mV for α1β2b) and not normalized to the Ba^{2+} equilibrium potential.

Fig. 5A shows representative α1, α1β2a, and α1β2b currents normalized to peak current. Mean half-decay times were 440.2 ± 36.1 msec (n = 5) (α1β2a) and 469 ± 31.9 msec (n = 4) (α1β2b) (Fig. 5B). Mean half-decay times for α1 currents (793.3 ± 13.3 msec, n = 3) were significantly longer (unpaired t test, p = 0.0004) than those for either α1β2a or α1β2b currents, which were not significantly different from each other (unpaired t test, p = 0.58). No cell transfected with the β2b subunit alone (n = 14) demonstrated voltage-activated inward Ba^{2+} currents.

(+)-[^{3}H]PN200–110 binding to transiently transfected HEK 293 cells. Fura-2 imaging and electrophysiological recordings demonstrated that the β2 subunit increased the activity and current magnitude of α1 subunit currents. To further examine the role of the β2 subunit in modulation of the α1 subunit, HEK 293 cells were transfected with the α1 and β2 subunit expression vectors and binding of a dihydropyridine ligand was determined. Binding of (+)-[^{3}H]PN200–110 to membranes of transiently transfected HEK 293 cells demonstrated a 10-fold increase in dihydropyridine binding in cells coexpressing α1 and β2 subunits (Fig. 6). In contrast, cells transfected with α1 subunits alone demonstrated a very low level of binding. Wild-type HEK 293 cells or cells transfected with either β2 subunit alone did not demonstrate specific (+)-[^{3}H]PN200–110 binding (data not shown).

Discussion

The two cloned mouse β subunit isoforms coded for alternate splice variants similar to the previously described rat and rabbit β2 cDNAs, but these two murine isoforms contained a previously uncharacterized amino terminus (25, 41). Both isoforms were primarily expressed in heart and brain, but lower levels were detected in lung, kidney, and testis. Although similar splice variants internal to the coding region have been described for the β2 subunit, the amino terminus found in both β2a and β2b (amino acids 1–17) has not been reported previously. The β2a and β2b subunits coded for cytoplasmic proteins that contain 611 and 572 amino acids, respectively, with predicted molecular weights of 68,930 for β2a and 64,670 for β2b. Both β2 isoforms contained a high α-helical content, as reported for other previously cloned β subunit isoforms (23).

Voltage-dependent Ca^{2+} channels, especially in skeletal and cardiac muscle, are modulated by phosphorylation mediated by protein kinases (42). Phosphorylation of both skeletal muscle α1 and β1 subunits has been demonstrated (43). One consensus sequence for cAMP-dependent protein kinase (Arg-Lys-Ser-Thr-171) was present in both β2a and β2b, as shown in Fig. 1. This phosphorylation site was conserved in the rat, rabbit, and human skeletal muscle β1 and cardiac/brain β2 subunits (25, 44). Both β2 subunits also contained several potential protein kinase C phosphorylation sites (Ser/Thr-X-Lys/Arg), as shown in Fig. 1. Previous work demonstrated that the β1 subunit was phosphorylated by cAMP- and cGMP-dependent protein kinases and protein kinase C (43, 45). Although not fully demonstrated in any study to

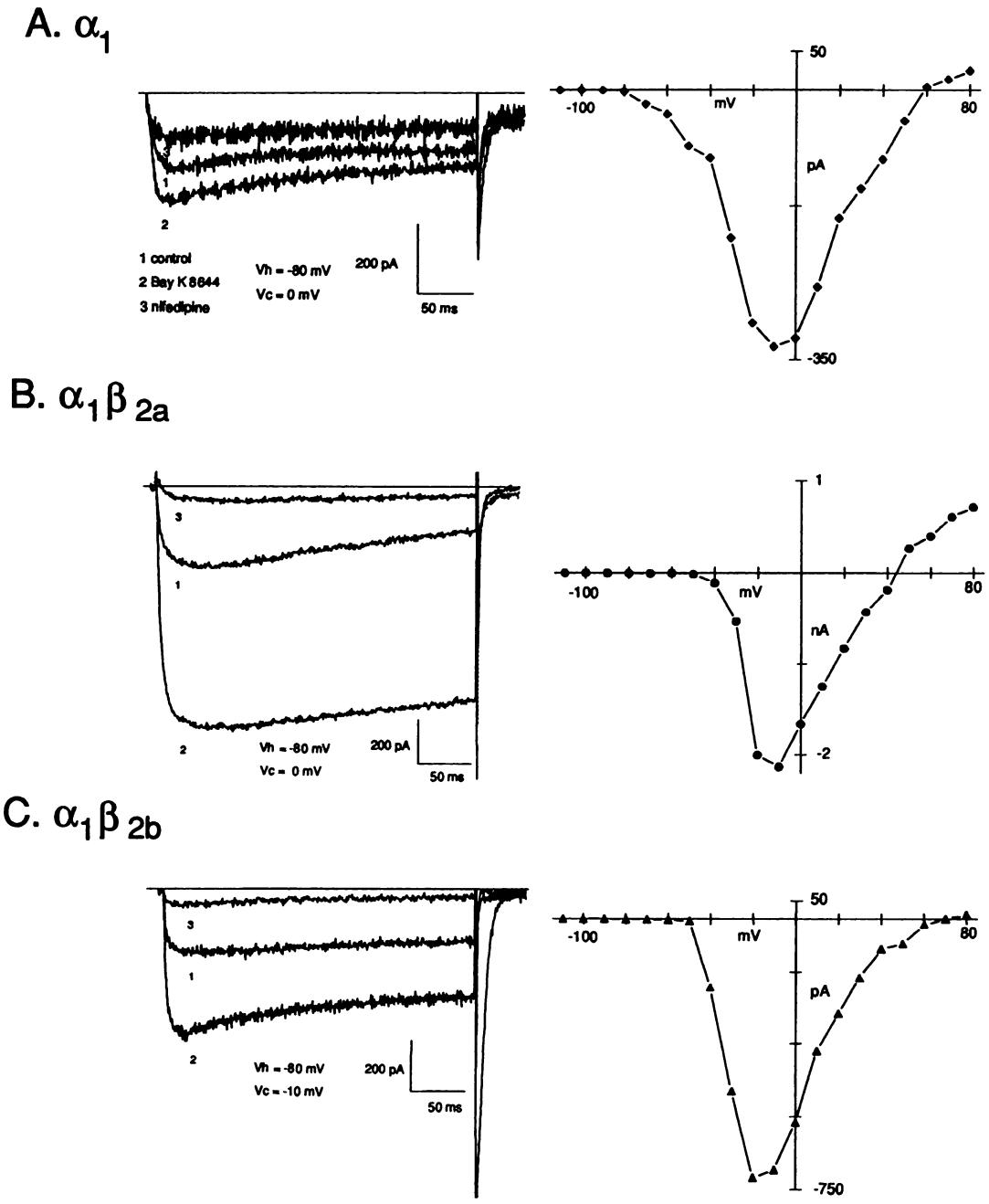

Fig. 4. Current traces and current-voltage relations for α1, α1β2a, and α1β2b subunits expressed in HEK 293 cells. *Left*, Ba^{2+} current traces for α1 (A), α1β2a (B), and α1β2b (C) subunit combinations, for control (*1*) and after application of Bay K 8644 (*2*) and nifedipine (*3*). Currents were evoked from a holding potential of −80 mV to clamp potentials of −10 or 0 mV as indicated. *Right*, current-voltage relations for α1 (A), α1β2a (B), and α1β2b (C) subunits. Peak currents occurred in the range of −20 to −10 mV.

date, phosphorylation-dependent modulation of Ca^{2+} channels may involve phosphorylation of the β subunit.

To demonstrate that the β2 cDNAs coded for functional β subunits, expression vectors coding for the full length β2a and β2b were constructed, using the human CMV promoter to direct their expression. The β subunit expression vectors were co-transfected with pCMV.MC1, the previously described mouse α1 expression vector (32). Because transient transfection resulted in a high incidence of coexpression of two different plasmids (35, 46), those cells that expressed both α1 and β2 subunits were predicted to form functional Ca^{2+} channels. Because Ca^{2+} entry into cells via Ca^{2+} channels results in increased $[Ca^{2+}]_i$, we used fura-2 imaging to demonstrate functional expression of the α1 and β2 subunits. Although fura-2 imaging has been used to detect functional Ca^{2+} channels in neurons, fura-2 imaging has not been used to detect functional expression of recombinant voltage-dependent Ca^{2+} channels. Fura-2 imaging of recombinant Ca^{2+} channels is advantageous because large numbers of transfected cells can be assayed in a relatively short time and this technique measures the actual change in $[Ca^{2+}]_i$, which mediates many cellular responses to calcium.

Transfection of cells with α1 and β2 subunits formed functional Ca^{2+} channels that were activated by Bay K 8644 and

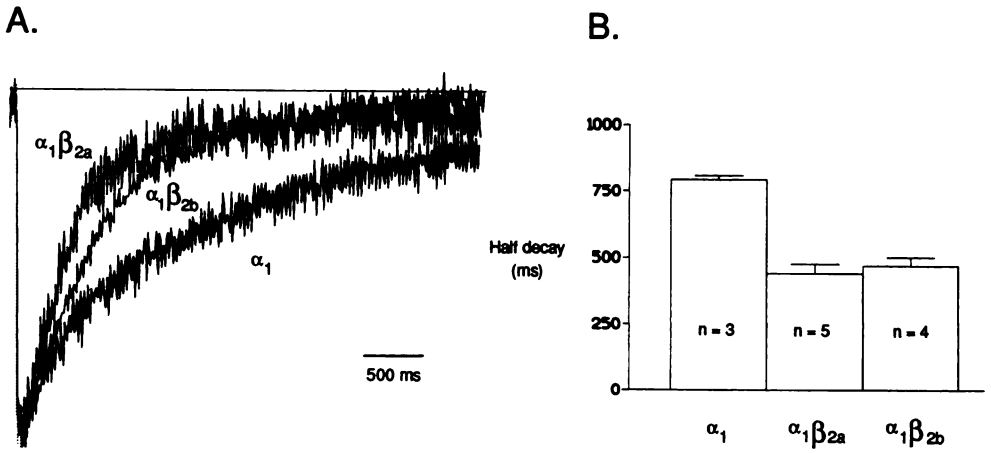

Fig. 5. Inactivation of currents evoked from $\alpha 1$, $\alpha 1\beta 2a$, and $\alpha 1\beta 2b$ subunits expressed in HEK 293 cells. A, Representative Ba^{2+} current traces from $\alpha 1$, $\alpha 1\beta 2a$, and $\alpha 1\beta 2b$ subunits normalized to peak current. Currents of similar magnitude for the different subunits were selected for comparison. Current inactivation for $\alpha 1\beta 2a$ and $\alpha 1\beta 2b$ subunits was faster than that for $\alpha 1$ subunits. B, Difference in the time to 50% current inactivation (half-decay time) for $\alpha 1$, $\alpha 1\beta 2a$, and $\alpha 1\beta 2b$ subunit combinations.

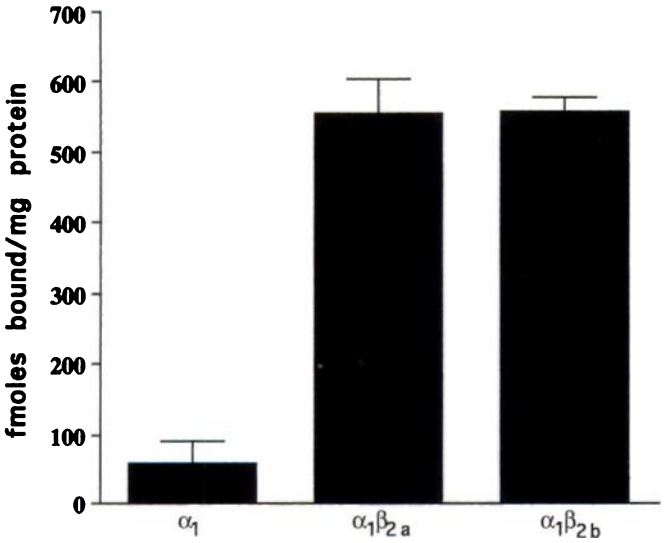

Fig. 6. Dihydropyridine binding to transiently transfected HEK 293 cell membranes. HEK 293 cells were transfected with the indicated subunit combinations and membranes were isolated after 48 hr. Membranes were incubated with (+)-[^{3}H]PN200–110. Total and nonspecific binding was determined in the absence and presence of nifedipine, respectively. Binding was normalized to β-galactosidase expression and protein concentration. Transfection efficiency among the different transfections was consistent and showed no significant difference. Data represent the mean ± standard deviation of a single transfection performed in triplicate. The binding was repeated twice for two separate transfections.

55.6 mM KCl. Nifedipine, a dihydropyridine antagonist, was able to inhibit the response to Bay K 8644 and 55.6 mM KCl. However, fura-2 imaging did not detect functional differences between the two $\beta 2$ subunit isoforms. Although previous electrophysiological analysis demonstrated that the class C cardiac $\alpha 1$ subunit formed functional Ca^{2+} channels when expressed in oocytes, fura-2 imaging experiments reported here did not detect expression of functional Ca^{2+} channels in cells transfected with the $\alpha 1$ subunit alone. Previously, Scatchard analysis of COS-1 cells transfected with the cardiac $\alpha 1$ subunit alone demonstrated the presence of high affinity binding sites, but electrophysiological recordings were not performed (25). Cells expressing both the rat cardiac $\alpha 1$ and $\beta 2$ subunits expressed channels that had the same affinity for dihydropyridines but the number of binding sites was greatly increased, in comparison with cells expressing only the $\alpha 1$ subunit (25). In addition, coexpression of $\alpha 1$ and β subunits in oocytes produced larger Ca^{2+} channel currents, compared with those in oocytes expressing only the $\alpha 1$ subunit (25). Thus, the inability of fura-2 imaging to detect functional channels in cells expressing the $\alpha 1$ subunit alone may reflect a smaller population of functional channels that may also generate much smaller currents. The absence of detectable calcium channels in COS-1 cells expressing only the $\alpha 1$ subunit, as well as the lack of differential effects of the two $\beta 2$ isoforms, prompted additional electrophysiological analysis of these calcium channel subunits.

To further examine the effect of the $\beta 2$ subunits on the $\alpha 1$ subunit, HEK 293 cells were transfected with expression vectors and then Ca^{2+} channel activity was measured by whole-cell, patch-clamp, electrophysiological techniques, using Ba^{2+} as the charge carrier. This work is the first transient expression of neuronal, class C-I, dihydropyridine-sensitive, Ca^{2+} channels in mammalian cells. One consideration when Ca^{2+} channel subunits are expressed in oocytes is the presence of an endogenous, high-threshold, dihydropyridine- and ω-conotoxin GVIA-insensitive Ca^{2+} current (26). In contrast to *Xenopus* oocytes, mammalian cell lines such as HEK 293 do not express endogenous Ca^{2+} channel subunits or currents (data not shown).

Transfection of cells with the $\alpha 1$ subunit alone or with either $\beta 2$ subunit expression vector led to the expression of high-threshold Ca^{2+} currents that were dihydropyridine sensitive (Fig. 4). Cells that were transfected with the $\beta 2b$ subunit alone or mock transfected did not express Ba^{2+} currents (data not shown).

The $\alpha 1$ subunit currents differed from the $\alpha 1\beta 2$ currents, and often $\alpha 1$ subunit currents were undetectable in the absence of Bay K 8644. However, several cells expressed larger currents than could be detected in the absence of Bay K 8644. Activation and inactivation kinetics of the larger $\alpha 1$ subunit currents were identical to those of the smaller currents, suggesting that the channels were identical. The $\alpha 1\beta 2$ subunit currents were of greater magnitude and were usually detected in the absence of Bay K 8644. Expression of either

β2 subunit isoform was sufficient to potentiate the current magnitude in HEK 293 cells. The previously described rat β2 subunit was demonstrated to increase the current magnitude 10-fold in oocytes expressing the rabbit cardiac α1 subunit (25). Activation kinetics of the mouse class C-I α1 subunit currents appeared to differ from those of the previously described cardiac, smooth muscle, and class C-II α1 subunit currents (25, 47, 48). The difference in activation kinetics for the class C-I α1 subunit currents may reflect the role of alternative splicing in the α1 subunit of voltage-dependent Ca^{2+} channels.

In comparison with α1 subunit currents, the activation time of α1β2 subunit currents was identical but the inactivation time was shorter. Similar alterations of Ca^{2+} channel inactivation kinetics have been observed for other β subunit classes in oocytes (28). The altered inactivation kinetics of the α1β2 subunit currents suggested that the β subunit plays several distinct roles in the modulation of the calcium channels. Alternative splicing of the β2 subunit does not seem to differentially affect the function of the recombinant Ca^{2+} channel currents.

Fura-2 imaging and electrophysiological recordings demonstrated that the presence of β2 subunits increased the current magnitude and inactivation rate of currents evoked from α1 subunits. Dihydropyridine binding demonstrated that the β2 subunit increased the expression of dihydropyridine binding in transfected HEK 293 cells. The large increase in dihydropyridine binding in cells coexpressing the α1 and β2 subunits may explain the absence of functional channels in fura-2-imaged COS-1 cells expressing only the α1 subunit, because the influx of calcium through α1 subunit channels may not increase $[Ca^{2+}]_i$ to levels detectable by fura-2 imaging. The increase in dihydropyridine binding also supported the observed increase in current magnitude when the β2 subunit was coexpressed with the α1 subunit in HEK 293 cells. The mechanism of increased expression is unknown, although recent reports suggested that the β subunit modulates the dihydropyridine sensitivity of the α1 subunit and does not alter the relative expression of the α1 subunit (49, 50).

These experiments are significant for several reasons. First, expression of β2 subunit isoforms with the previously uncharacterized class C-I α1 subunit demonstrated the effect of β2 subunit coexpression on α1 subunit currents and suggests that β2 subunit effects are relatively independent of the α1 subunit isoform that is coexpressed. In contrast to previous reports, the β2 subunit did not alter the activation rate of α1 subunit currents. However, the β2 subunit increased the inactivation rate of the α1 subunit currents. Coexpression of either the β2a or β2b subunit isoform increased the magnitude of the α1 subunit currents. Second, no distinct functional differences between the two β2 subunits were detected. The alternative splicing of the β subunit may suggest a distinct role for these subunit isoforms in cellular localization or interaction with other accessory or modulatory proteins. Finally, the expression of these α1 and β subunit isoforms in mammalian cells is significant because previous expression of many neuronal Ca^{2+} channels has been achieved mainly by using *Xenopus* oocytes. In contrast to oocytes, the expression of Ca^{2+} channel subunits in mammalian cell lines such as COS-1 or HEK 293 permitted both fura-2 imaging and electrophysiological characterization of recombinant Ca^{2+} channels in the absence of endogenous Ca^{2+} channel currents or subunits.

The α1 and β2 subunit expression vectors should be useful for further characterization of the modulatory roles of the β subunit, especially when used in a rapid screening assay such as fura-2 imaging. Functional analysis of uncharacterized recombinant calcium channels would also be facilitated. Initial rapid screening would permit the optimization of transfection conditions and determination of pharmacological properties of the expressed currents without alterations of the intracellular environment. Detailed analysis could then be performed using electrophysiological recordings.

Furthermore, the comparison of electrophysiological properties of recombinant Ca^{2+} channels with experimentally determined $[Ca^{2+}]_i$ values may allow more detailed studies of the specific role of calcium entry via voltage-sensitive Ca^{2+} channels in the regulation of important cellular processes such as enzyme regulation and gene trascription. In addition to studies of the regulation of cellular processes, imaging and electrophysiological analysis should help to elucidate the regulation of recombinant Ca^{2+} channels by protein kinase-dependent phosphorylation.

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