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# HUMAN NEURONAL VOLTAGE-DEPENDENT CALCIUM CHANNELS: STUDIES ON SUBUNIT STRUCTURE AND ROLE IN CHANNEL ASSEMBLY

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**Summary**—Voltage-dependent calcium ($Ca^{2+}$) channels, expressed in the CNS, appear to be multimeric complexes comprised of at least $\alpha_1$, $\alpha_2$ and $\beta$ subunits. Previously, we cloned and expressed human neuronal $\alpha_1$, $\alpha_2$ and $\beta$ subunits to study recombinant channel complexes that display properties of those expressed *in vivo*. The $\alpha_{1B}$-mediated channel subtype binds $\omega$-conotoxin (CgTx) GVIA with high affinity and exhibits properties of N-type voltage-dependent $Ca^{2+}$ channels. Here we describe several $\alpha_2$ and $\beta$ splice variants and report results on the expression of $\omega$-CgTx GVIA binding sites, assembly of the subunit complex and biophysical function of $\alpha_{1B}$-mediated channel complexes containing some of these splice variants. We optimized recombinant expression in human embryonic kidney (HEK) 293 cells of $\alpha_{1B}\alpha_{2b}\beta_1$ subunit complexes by controlling the expression levels of subunit mRNAs and monitored cell surface expression by binding of $\omega$-CgTx GVIA to the $\alpha_{1B}$ subunit. Co-expression of either $\alpha_{2b}$ or $\beta_1$ subunits with an $\alpha_{1B}$ subunit increased expression of binding sites while the most efficient expression was achieved when both $\alpha_{2b}$ and $\beta_1$ subunits were co-expressed with an $\alpha_{1B}$ subunit. The presence of $\alpha_{2b}$ affects the affinity of $\omega$-CgTx GVIA binding and barium ($Ba^{2+}$) current magnitudes, although it does not appear to alter kinetic properties of the $Ba^{2+}$ current. This is the first evidence of an $\alpha_2$ subunit modulating the binding affinity of a cell-surface $Ca^{2+}$ channel ligand. Our results demonstrate that $\alpha_1$, $\alpha_2$ and $\beta$ subunits together contribute to the efficient assembly and functional expression of voltage-dependent $Ca^{2+}$ channel complexes.

*Key words*—$\alpha_2$ subunit, $\beta$ subunit, $\omega$-conotoxin GVIA, cloning, expression, function.

Intracellular $Ca^{2+}$ levels mediate a variety of events in neuronal cells including neurosecretion, neurotransmitter release and gene expression. Voltage-dependent $Ca^{2+}$ channels are multiple subunit membrane complexes that regulate the entry of $Ca^{2+}$ into excitable cells. In the rabbit skeletal muscle, four tightly coupled subunits, $\alpha_1$, $\alpha_2$, $\beta$ and $\gamma$ comprise the channel complex (see Campbell, Leung and Sharp, 1988, for review). Biochemical evidence and recent studies with cloned subunit gene sequences suggest that voltage-dependent $Ca^{2+}$ channel complexes in the CNS are comprised of at least $\alpha_1$, $\alpha_2$ and $\beta$ subunits (see Snutch and Reiner, 1992, for review; Williams, Brust, Feldman, Patthi, Simerson, Maroufi, McCue, Veliçelebi, Ellis and Harpold, 1992a), whereas transcripts encoding a $\gamma$ subunit have not been identified in RNA from brain (Jay, Ellis, McCue, Williams, Vedvick, Harpold and Campbell, 1990; Bosse, Regulla, Biel, Ruth, Meyer, Flockerzi and Hofmann, 1990). The $\alpha_1$ and $\beta$ subunits are each encoded by a gene family, including at least six distinct genes for $\alpha_1$ subunits and four genes for $\beta$ subunits (Williams *et al.*, 1992a, and references therein; Niidome, Kim, Friedrich and Mori, 1992; Castellano, Wei, Birnbaumer and Perez-Reyes, 1993b). Primary transcripts of each of the $\alpha_1$ genes, the $\alpha_2$ gene and two of the $\beta$ genes have been shown to yield multiple, structurally distinct subunits via differential mRNA processing (see Tsien, Ellinor and Horne, 1991, for review; Williams *et al.*, 1992a; Hullin, Singer-Lahat, Freichel, Biel, Dascal, Hofmann and Flockerzi, 1992; Williams, Feldman, McCue, Brenner, Veliçelebi, Ellis and Harpold, 1992b; Pragnell, Sakamoto, Jay and Campbell, 1991; Perez-Reyes, Castellano, Kim, Bertrand, Baggstrom, Lacerda, Wei and Birnbaumer, 1992; Kim, Kim, Lee, King and Chin, 1992).

Initial reports of functional recombinant voltage-dependent $Ca^{2+}$ channels indicated that an $\alpha_1$ subunit forms the pore through which $Ca^{2+}$ enters the cell (Mikami, Imoto, Tanabe, Niidome, Mori, Takeshima, Narumiya and Numa, 1989; Perez-Reyes, Kim, Lacerda, Horne, Wei, Rampe, Campbell, Brown and Birnbaumer, 1989). After rabbit skeletal muscle $\alpha_2$, $\beta$ and $\gamma$ cDNA clones became available, these subunits were expressed in *Xenopus* oocytes in all possible combinations with rabbit neuronal $\alpha_{1A}$ (Mori, Friedrich, Kim, Mikami,

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Nakai, Ruth, Bosse, Hofmann, Flockerzi, Furuichi, Mikoshiba, Imoto, Tanabe and Numa, 1991) or rabbit cardiac $\alpha_{1C}$ subunits (Singer, Biel, Lotan, Flockerzi, Hofmann and Dascal, 1991) in order to determine their functional contribution to the channel complex. In these studies each subunit co-expressed with an $\alpha_1$ subunit altered the magnitude and/or other properties of the current. While these results suggest that $\alpha_2$, $\beta$ and $\gamma$ subunits could also have important functional roles in $Ca^{2+}$ channel properties, the conclusions are limited because additional studies indicate that these subunit forms are not co-expressed in the same cells or tissues *in vivo*.

To study the pharmacology and physiology of recombinant human neuronal voltage-dependent $Ca^{2+}$ channels that display the properties of channels expressed *in vivo*, we have cloned and expressed gene sequences encoding human neuronal $\alpha_1$, $\alpha_2$ and $\beta$ subunits (Williams *et al.*, 1992a, b). Our results suggested that maximal expression of channel complexes on the cell surface requires co-expression of all three subunits. We and others have reported the tissue-specific processing of the $\alpha_2$ and $\beta_1$ primary transcripts, for example, $\alpha_{2a}$ and $\beta_{1\text{-}1}$ both appear to be exclusively expressed in skeletal muscle (Williams *et al.*, 1992b; Kim *et al.*, 1992; Powers, Lui, Hogan and Gregg, 1992). We report here the DNA sequence characterization of several human $\alpha_2$ and $\beta_1$ splice variants.

We previously described the cloning and co-expression of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ subunits encoding an $\omega$-CgTx GVIA-sensitive human N-type $Ca^{2+}$ channel (Williams *et al.*, 1992a). The existence of multiple neuronal forms of $\alpha_{1B}$ and $\beta_1$ (Williams *et al.*, 1992b) $Ca^{2+}$ channel subunits, in addition to a neuronal-specific $\alpha_{2b}$ (Williams *et al.*, 1992b), suggests that neuronal heteromeric $Ca^{2+}$ channel subtypes might exhibit different pharmacological and physiological properties. We now have extended our previous studies by determining conditions for efficient cell surface expression of $\alpha_{1B}$-mediated $\omega$-CgTx GVIA receptors in HEK293 cells. These studies demonstrate that the number of $\omega$-CgTx GVIA receptors assembled and expressed on the cell surface is determined by both the subunit combination and the $\alpha_{1B}$ and $\beta_1$ splice variants that are co-expressed. Furthermore, the $\alpha_{2b}$ subunit affects the affinity of $\omega$-CgTx GVIA binding and $Ba^{2+}$ current magnitudes, although it does not appear to alter kinetic properties of the $Ba^{2+}$ current.

## METHODS

### *Nomenclature*

The following nomenclature is used for the $\alpha_1$, $\alpha_2$ and $\beta$ genes and isoforms of these genes. For the $\alpha_1$ gene family, we have adapted the nomenclature used for cDNAs originally described in rat brain (Snutch, Leonard, Gilbert, Lester and Davidson, 1990). Thus, the classes A, B, C and D are designated $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$ and $\alpha_{1D}$. A fifth gene has been recently described, $\alpha_{1E}$ (BII; Niidome *et al.*, 1992). The $\alpha_1$ gene expressed in skeletal muscle (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose and Numa, 1987) is named $\alpha_{1skm}$. Isoforms are named numerically, e.g. $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$. For the $\beta$ gene family, the nomenclature of Hullin *et al.* (1992) has been adapted. Thus CaB1, CaB2 and CaB3 are designated $\beta_1$, $\beta_2$ and $\beta_3$. Isoforms are named numerically, e.g. $\beta_{1\text{-}1}$, $\beta_{1\text{-}2}$ etc. The $\beta_1$ gene product identified in skeletal muscle (Ruth, Röhrkasten, Biel, Bosse, Regulla, Meyer, Flockerzi and Hofmann, 1989) is named $\beta_{1\text{-}1}$. The nomenclature for the $\alpha_2$ gene was described previously (Williams *et al.*, 1992b).

### *PCR analysis*

PCR reactions were performed as described previously (Williams *et al.*, 1992b). Human primary tissue (skeletal muscle, hippocampus/basal ganglia and aorta) the HEK293 cell poly(A)$^+$ RNAs were used as templates to synthesize cDNA prior to PCR analysis. PCR products were cloned into pCRII (Invitrogen, San Diego, California) and their DNA sequence was determined.

### *Transfections*

HEK293 cells were maintained in DMEM (Gibco #320-1965AJ), 5.5% Defined/Supplemented bovine calf serum (Hyclone #A-2151-L), 100 U/ml penicillin G and 100 $\mu$g/ml streptomycin. $Ca^{2+}$-phosphate based transient transfections were performed and analyzed as described previously (Williams *et al.*, 1992a). Cells were co-transfected with either 8 $\mu$g pcDNA1$\alpha_{1B\text{-}1}$ (Williams *et al.*, 1992a), 5 $\mu$g pHB-CaCH$\alpha_{2b}$(A) (Williams *et al.*, 1992b), 2 $\mu$g pHB-CaCH$\beta_{2b}$ · RBS(A) ($\beta_{1\text{-}2}$ expression plasmid; Williams *et al.*, 1992b) and 2 $\mu$g pCMV$\beta$-gal (Clontech, Palo Alto, California) (2:1.8:1 molar ratio of $Ca^{2+}$ channel subunit expression plasmids) or with 3 $\mu$g pcDNA1$\alpha_{1B\text{-}1\text{ or }2}$, 11.25 $\mu$g pHBCaCH$\alpha_{2b}$(A), 0.75 or 1.0 $\mu$g pHBCaCH$\beta_{2b}$ · RBS(A) or pcDNA1$\beta_{1\text{-}3}$ and 2 $\mu$g pCMV$\beta$-gal (2:10.9:1 molar ratio of $Ca^{2+}$ channel subunit expression plasmids). pCMV$\beta$-gal, a $\beta$-galactosidase expression plasmid, was included in the transfections as a marker to permit transfection efficiency estimates by histochemical staining. When less than three subunits were expressed, pCMVPL2, a pCMV promoter-containing vector that lacks a cDNA insert, was substituted to maintain equal moles of pCMV-based DNA in the transfection. pUC18 DNA was used to maintain the total mass of DNA in the transfection at 20 $\mu$g/plate.

### *mRNA isolation and analysis*

Northern blot hybridization analysis was performed on total mRNA extracted from untransfected and transfected HEK293 cells. $Ca^{2+}$ channel subunit mRNAs were detected with random-primed $^{32}$P-labeled subunit-specific cDNA probes.

*[$^{125}$I]ω-CgTx GVIA binding to transfected cells*

The specific binding of ω-CgTx GVIA to transfected HEK293 cells was determined as described previously (Williams *et al.*, 1992a). Statistical analysis of the $K_d$ and $B_{max}$ values was performed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple pairwise comparisons ($P \leq 0.05$).

*Electrophysiology*

Functional expression of $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ and $\alpha_{1B\text{-}1}\beta_{1\text{-}2}$ subunit combinations was evaluated using the whole-cell recording technique as previously described (Williams *et al.*, 1992a). Transfected cells that had no contacts with surrounding cells and simple morphology were used approximately 48 hours after transfection for recording. The pipette solution was (in mM) 135 CsCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES and 4 mM Mg-ATP (pH 7.3, adjusted with TEA-OH). The external solution was (in mM) 15 $BaCl_2$, 125 Choline Cl, 1 $MgCl_2$ and 10 HEPES (pH 7.3, adjusted with TEA-OH). ω-CgTx GVIA (Bachem) was prepared in the external solution with 0.1% cytochrome C (Sigma) to serve as a carrier. Control experiments showed that cytochrome C had no effect on the $Ba^{2+}$ current.

## RESULTS

*Differential processing of the $\alpha_2$ and $\beta_1$ primary transcripts*

Previously, we characterized the $\alpha_{2b}$ and $\beta_{1\text{-}2}$ splice variants of human neuronal voltage-dependent $Ca^{2+}$ channels and compared the deduced amino acid sequences to the rabbit skeletal muscle $\alpha_{2a}$ and $\beta_{1\text{-}1}$ subunits (Williams *et al.*, 1992b). Comparison of the rabbit $\alpha_{2a}$ and human $\alpha_{2b}$ deduced amino acid sequences revealed two regions of notable divergence: a 19-amino acid deletion in the $\alpha_{2b}$ sequence compared with the $\alpha_{2a}$ sequence ($\alpha_{2a}$ residues Pro-507 to Gln-525) and a 7-amino acid insertion in the $\alpha_{2b}$ sequence compared with the $\alpha_{2a}$ sequence ($\alpha_{2b}$ residues Lys-602 to Asp-608). PCR and DNA hybridization analyses of human tissue-specific $\alpha_2$ transcripts revealed three splice variants in the 19- and 7-amino acid regions: $\alpha_{2a}$ (540 bp), $\alpha_{2b}$ (501 bp) and $\alpha_{2c}$ (490 bp).

We now have cloned and sequenced tissue-specific $\alpha_2$ PCR products through the 19- and 7-amino acid-encoding regions of divergence and have identified five $\alpha_2$ splice variants (Fig. 1). The results of the DNA sequence analysis suggest that three $\alpha_2$ regions are differentially processed to produce $\alpha_{2a}$ (encodes regions 1 and 2), $\alpha_{2b}$ (encodes regions 2 and 3), $\alpha_{2c}$ (encodes region 3), $\alpha_{2d}$ (does not encode regions 1, 2 or 3) and $\alpha_{2e}$ (encodes region 2).

A putative splice acceptor CAG trinucleotide exists at the 3′ end of regions 1 and 2 (Fig. 1, encodes Gln-525 and Gln-530) which suggests that differential processing of these regions results from alternative use of splice acceptor sequences. The third region is most likely encoded by a single exon because there are no consensus splice sites present in the $\alpha_{2b}$ and $\alpha_{2c}$ forms compared to the forms that lack region 3.

We previously analyzed human habenula, thalamus, hippocampus/basal ganglia, aorta, skeletal muscle and human neuroblastoma IMR32 RNAs for $\alpha_2$ expression (Williams *et al.*, 1992b). Based on the size of the PCR products, the $\alpha_{2a}$ transcript is expressed in skeletal muscle, the $\alpha_{2b}$ transcript is expressed in the CNS tissues and IMR32 cells, and the $\alpha_{2c}$ transcript is expressed in aorta tissue. In addition to this distribution, we now have identified $\alpha_{2d}$ and $\alpha_{2e}$ RNAs in aorta tissue and, further, $\alpha_{2d}$ and $\alpha_{2e}$ are expressed in HEK293 cells.

Analogous to the $\alpha_2$ deduced amino acid sequence comparison, a comparison of the rabbit skeletal muscle ($\beta_{1\text{-}1}$) and human neuronal ($\beta_{1\text{-}2}$) deduced amino acid sequences revealed a region of heterogeneity [human $\beta_{1\text{-}2}$ residues Ala-210 to Thr-217; Fig. 2(A)]. PCR analysis of RNAs isolated from human habenula, thalamus, hippocampus/basal ganglia, aorta, skeletal muscle and IMR32 cells through the region of heterogeneity revealed that $\beta_{1\text{-}1}$ is expressed in skeletal muscle tissue, transcripts containing a proposed exon present in both $\beta_{1\text{-}2}$ and $\beta_{1\text{-}3}$ are expressed in CNS regions and IMR32 cells, and $\beta_{1\text{-}4}$ is expressed in aorta tissue (Williams *et al.*, 1992b). We now have determined the complete primary structure of $\beta_{1\text{-}3}$ and identified an additional splice variant, $\beta_{1\text{-}5}$, which is expressed in HEK293 cells. As shown in Fig. 2(A), $\beta_{1\text{-}3}$ diverges from $\beta_{1\text{-}2}$ at amino acid 445 and the $\beta_{1\text{-}3}$ carboxyl terminus extends an additional 120 amino acids. $\beta_{1\text{-}3}$ encodes the human homolog of the previously reported rat neuronal $\beta_1$ splice variant (Pragnell *et al.*, 1991). A further comparison of these splice variants is shown in Fig. 2(B). Interestingly, the $\beta_{1\text{-}4}$ and $\beta_{1\text{-}5}$ variants appear to result from aberrant splicing of the $\beta$ primary transcript that includes a guanine nucleotide which disrupts the reading frame. Recently, the structure of the human $\beta$ gene through this region was reported (Powers *et al.*, 1992) and confirmed this aberrant splicing. Powers *et al.* (1992) showed that in the human genome the skeletal muscle-specific sequence and a neuronal-specific sequence (Fig. 2) are encoded by separate exons. One of these two exons must be included in the processing in order to maintain the proper reading frame. Neither $\beta_{1\text{-}4}$ nor $\beta_{1\text{-}5}$ contains these exons. In addition, $\beta_{1\text{-}5}$ lacks a third exon, which Powers *et al.* (1992) characterized as "common" between the skeletal muscle and neuronal splice variants. It is not yet known whether $\beta_{1\text{-}4}$ and $\beta_{1\text{-}5}$ produce functional proteins.

*Recombinant expression of human neuronal ω-CgTx GVIA-sensitive voltage-dependent $Ca^{2+}$ channels*

An important consideration regarding the pharmacological and physiological study of recombinant voltage-dependent $Ca^{2+}$ channels is the co-expression of all of the subunits that comprise the $Ca^{2+}$ channel

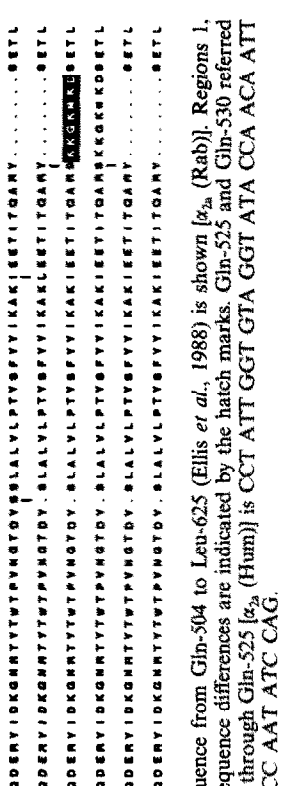

Fig. 1. Deduced amino acid sequence alignment of $\alpha_2$ splice variants. The rabbit skeletal muscle sequence from Gln-504 to Leu-625 (Ellis *et al.*, 1988) is shown [$\alpha_{2a}$ (Rab)]. Regions 1, 2 and 3 are indicated at the top and also shown as white letters on black background. Amino acid sequence differences are indicated by the hatch marks. Gln-525 and Gln-530 referred to in the text are indicated by the *. The human skeletal muscle DNA sequence that encodes Pro-507 through Gln-525 [$\alpha_{2a}$ (Hum)] is CCT ATT GGT GTA GGT ATA CCA ACA ATT AAT TTA AGA AAA AGG AGA CCC AAT ATC CAG.

*in vivo*. cDNA clones that encode functional $\alpha_1$ subunits have been isolated from rabbit skeletal muscle ($\alpha_{1skm}$; Perez-Reyes *et al.*, 1989), rabbit heart ($\alpha_{1C}$; Mikami *et al.*, 1989), rabbit brain ($\alpha_{1A}$; Mori *et al.*, 1991), human brain ($\alpha_{1D}$ and $\alpha_{1B}$; Williams *et al.*, 1992a, b) and rat brain ($\alpha_{1B}$; Soong, Stea, Hodson, Dubel, Vincent and Snutch, 1993). Several of these studies were performed with incomplete channel complexes containing $\alpha_1$ subunits but lacking other subunits known to be associated with voltage-dependent $Ca^{2+}$ channels, e.g. $\alpha_2$ and $\beta$ subunits (Perez-Reyes *et al.*, 1992 and references therein; Lory, Varadi, Slish, Varadi and Schwartz, 1993; Bosse, Bottlender, Kleppisch, Hescheler, Welling, Hofmann and Flockerzi, 1992; Castellano, Wei, Birnbaumer and Perez-Reyes, 1993a; Mikami *et al.*, 1989; Soong *et al.*, 1993). Other studies involve artificial channel complexes comprised of non-skeletal muscle $\alpha_1$ subunits ($\alpha_{1A}$ or $\alpha_{1C}$) and skeletal muscle $\alpha_2$ ($\alpha_{2a}$) and/or $\beta_1$ ($\beta_{1\text{-}1}$) subunits (Singer *et al.*, 1991; Mori *et al.*, 1991; Hullin *et al.*, 1992). Co-expression of different $\alpha_2$ and $\beta$ gene products with $\alpha_1$ genes might change the properties of the channel complex. For example, different $\beta$ gene products co-expressed with rabbit heart $\alpha_{1C}$ and rabbit skeletal muscle $\alpha_{2a}$ dramatically altered the electrophysiological and pharmacological properties of the channel complex (Hullin *et al.*, 1992). Similarly, in the neuromuscular nicotinic acetylcholine receptor, seemingly minor differences in the primary structure of the $\alpha$ and $\epsilon$ subunits dramatically alter the surface expression of the heteromeric protein complex (Gu, Camacho, Gardner and Hall, 1991; Chavez, Maloof, Beeson, Newsom-Davis and Hall, 1992). For reasons such as these, we have emphasized the cloning and expression of *human neuronal* $Ca^{2+}$ channel subunits, including $\alpha_{1D}$, $\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}2}$, $\alpha_{2b}$, $\beta_{1\text{-}2}$ and $\beta_{1\text{-}3}$ (Williams *et al.*, 1992a, b, and this work). We have shown that transcripts encoding these subunits are co-expressed in different regions of human brain as well as in IMR32 cells, a clonal human neuroblastoma cell line (Williams *et al.*, 1992b; unpublished results and above). Although we are not certain that transcripts encoding these subunits in human neuronal tissues produce channel complexes expressed *in vivo*, we can be sure that pharmacological and physiological differences we discover are not due to co-expression of neuronal and non-neuronal subunits.

Another important consideration regarding the expression of recombinant $Ca^{2+}$ channels is the endogenous expression of $Ca^{2+}$ channel subunit proteins in the host cell. Although we have detected $\alpha_2$ and aberrant $\beta_1$ PCR products in HEK293 cells, we cannot detect $\alpha_1$, $\alpha_2$ or $\beta_1$ mRNAs by Northern hybridization analysis [Fig. 3, lanes (1)] and do not detect significant $Ba^{2+}$ currents in untransfected HEK293 cells. Furthermore, HEK293 cells do not express detectable $\omega$-CgTx GVIA binding sites in either untransfected cells or cells transfected with plasmids other than $\alpha_1$, i.e. $\alpha_{2b}$ and/or $\beta_1$. These

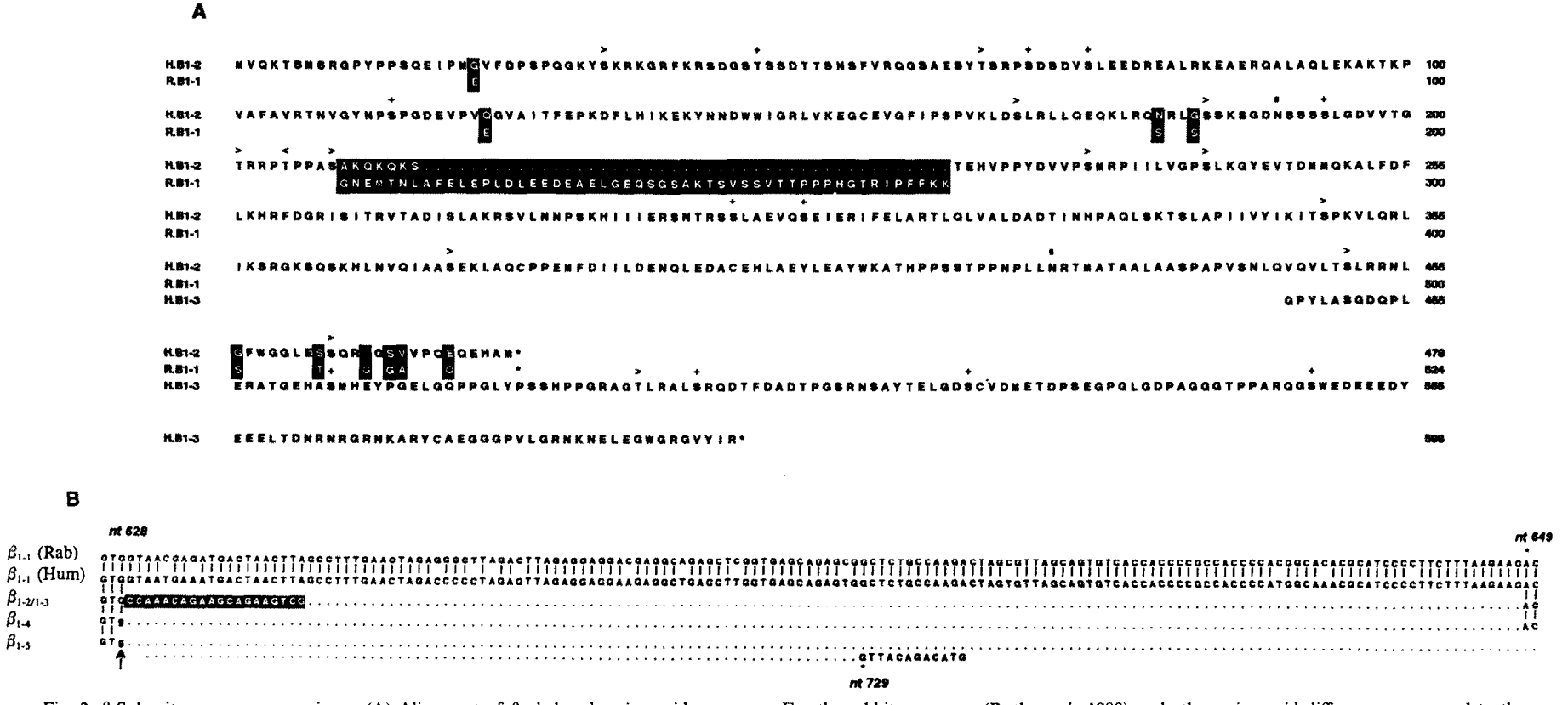


Fig. 2. $\beta$ Subunit sequence comparisons. (A) Alignment of $\beta_1$ deduced amino acid sequences. For the rabbit sequences (Ruth *et al.*, 1989), only the amino acid differences compared to the human sequences (H.B1-2) are shown. Sequence differences and insertions/deletions are identified by the black boxes. Only the human $\beta_{1-3}$ sequence (H.B1-3) that differs from $\beta_{1-2}$ is shown. Ser-450 and Ser-464 are potential protein kinase C phosphylation sites in $\beta_{1-2}$ but are not potential sites in $\beta_{1-3}$. Potential *N*-glycosylation (o), cyclic AMP-dependent phosphorylation ( < ), protein kinase C phosphorylation ( > ) and casein kinase II phosphorylation ( + ) sites are shown. (B) $\beta_1$ Nucleotide sequence through the region of heterogeneity. Human neuronal $\beta_{1-2}$ nt 628, 649 and 729 (Williams *et al.*, 1992b) are indicated with an * for reference. The arrow marks the guanine nucleotide that is not removed during processing (see text and Powers *et al.*, 1992).

observations are in contrast to the *Xenopus* oocyte host expression system. *Xenopus* oocytes do express endogenous $Ca^{2+}$ channels (Dascal, Snutch, Lübbert, Davidson and Lester, 1986; Singer *et al.*, 1991; Williams *et al.*, 1992b) and they express an uncharacterized, abundant $\alpha_2$ mRNA (Singer-Lahat, Lotan, Itagaki, Schwartz and Dascal, 1992). Moreover, human $\beta_{1\text{-}2}$ *in vitro* transcripts induced a detectable voltage-dependent $Ca^{2+}$ current that was pharmacologically and electrophysiologically distinguishable from human $\alpha_{1D}$-mediated currents, although it might not be distinguishable from other recombinant subtypes (Williams *et al.*, 1992b).

The optimized expression of $\alpha_{1B}$-mediated $\omega$-CgTx GVIA binding sites coupled with the insignificant voltage dependent $Ca^{2+}$ channel expression in HEK293 cells provides a very good system in which to examine $\alpha_{1B}$, $\alpha_{2b}$ and $\beta_1$ subunit combinations expressed transiently. Transient expression of the $Ca^{2+}$ channel subunits avoids selection pressure against subunits that may occur when stable expression is attempted (Varadi, Lory, Schultz, Varadi and Schwartz, 1991; Lacerda, Kim, Ruth, Perez-Reyes, Flockerzi, Hofmann, Birnbaumer and Brown, 1991; Lory, Varadi and Schwartz, 1992). The high efficiency of transfection (>50%) also enabled us to effectively analyze the macroscopic current characteristics of individual cells transfected with $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ and $\alpha_{1B\text{-}1}\beta_{1\text{-}2}$ subunit combinations.

To study the physiology and pharmacology of recombinant human neuronal voltage-dependent $Ca^{2+}$ channels more accurately, we have altered the $\alpha_{1B}$, $\alpha_{2b}$ and $\beta_1$ expression levels in order to approximate the expression of a homogeneous population of trimeric $\alpha_{1B}$, $\alpha_{2b}$ and $\beta_1$ protein complexes in transfected HEK293 cells. The efficiency of expression and assembly of channel complexes at the cell surface were optimized by adjusting the molar ratio of $\alpha_{1B}$, $\alpha_{2b}$ and $\beta_1$ expression plasmids used in the transfections. The transfectants were analyzed for mRNA levels, $\omega$-CgTx GVIA binding and $Ca^{2+}$ channel current density in order to determine near optimal channel expression in the absence of immunological reagents for evaluating protein expression.

### *mRNA analysis*

Although $\omega$-CgTx GVIA binding and current density analyses showed that each subunit contributed to functional $Ca^{2+}$ channel complexes (Williams *et al.*, 1992a), HEK293 cells co-transfected with $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ expression plasmids (8, 5 and 2 $\mu$g, respectively; molar ratio = 2:1.8:1) did not express equivalent levels of each $Ca^{2+}$ channel subunit mRNA [Fig. 3, lanes (2)]. Relatively high levels of $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ mRNAs were expressed, but significantly lower levels of $\alpha_{2b}$ mRNA were expressed. Based on autoradiograph exposures required to produce equivalent signals for all three mRNAS, $\alpha_{2b}$ transcript levels were estimated to be 5–10 times lower than $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ transcript levels. Untransfected HEK293 cells did not express detectable levels of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$, or $\beta_{1\text{-}2}$ mRNAs.

To achieve equivalent $Ca^{2+}$ channel subunit mRNA expression levels, a series of transfections was performed with various amounts of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ expression plasmids (data not shown). Because the $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ mRNAs were expressed at very high levels compared to $\alpha_{2b}$ mRNA [Fig. 3, lanes (2)], we lowered the mass of $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ plasmids and increased the mass of $\alpha_{2b}$ plasmid in the transfection experiments. Co-transfection with 3, 11.25 and 0.75 $\mu$g of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ expression plasmids, respectively (molar ratio = 2:10.9:1), approached equivalent expression levels of each $Ca^{2+}$ channel subunit mRNA [Fig. 3, lanes (3)]. The relative molar quantity of $\alpha_{2b}$ expression plasmid to $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ expression plasmids was increased 6-fold. The mass of $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ plasmids in the transfection was *decreased* 2.67-fold and the mass of $\alpha_{2b}$ plasmid was *increased* 2.25-fold. The 6-fold molar increase of $\alpha_{2b}$ relative to $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ required to achieve near equal abundance mRNA levels is consistent with the previous 5- to 10-fold lower estimate of relative $\alpha_{2b}$ mRNA abundance [Fig. 3, lanes (2)]. $\omega$-CgTx GVIA binding to cells transfected with various amounts of expression plasmids indicated that the 3, 11.25 and 0.75 $\mu$g of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ plasmids, respectively, improved the level of cell surface expression of channel complexes (see Discussion). Further increases in the mass of $\alpha_{2b}$ and $\beta_{1\text{-}2}$ expression plasmids while $\alpha_{1B\text{-}1}$ was held constant, and alterations in the mass of the $\alpha_{1B\text{-}1}$ expression plasmid while $\alpha_{2b}$ and $\beta_{1\text{-}2}$ were held constant, indicated that the cell surface expression of $\omega$-CgTx GVIA binding sites per cell was nearly optimal (unpublished observations; see Discussion). All subsequent transfections described below were performed with 3, 11.25 and 0.75 $\mu$g or 1.0 $\mu$g of $\alpha_{1B\text{-}1}$ or $\alpha_{1B\text{-}2}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ or $\beta_{1\text{-}3}$ expression plasmids, respectively.

### *$\omega$-CgTx GVIA binding*

Combinations of human voltage-dependent $Ca^{2+}$ channel subunits, $\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}2}$, $\alpha_{2b}$, $\beta_{1\text{-}2}$ and $\beta_{1\text{-}3}$, were analyzed for saturation binding of [$^{125}$I]$\omega$-CgTx GVIA. The transfected cells displayed a single-class of saturable, high affinity binding sites. Representative saturation curves and Scatchard plots are shown in Fig. 4 for HEK293 cells transfected with the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$, $\alpha_{1B\text{-}1}\beta_{1\text{-}3}$, $\alpha_{1B\text{-}1}\alpha_{2b}$ or $\alpha_{1B\text{-}1}$ subunit cDNAs. The values for the dissociation constants ($K_d$) and binding capacities ($B_{max}$) determined for the different combinations are summarized in Table 1. Cells transfected with subunit combinations lacking either the $\alpha_{1B\text{-}1}$ or the $\alpha_{1B\text{-}2}$ subunit did not exhibit any detectable [$^{125}$I]$\omega$-CgTx GVIA binding ($\leqslant$600 sites/cell; unpublished observation). In addition [$^{125}$I]$\omega$-CgTx GVIA binding to HEK293 cells transfected with $\alpha_{1B\text{-}2}$ alone or $\alpha_{1B\text{-}2}\alpha_{2b}$ was too low for reliable Scatchard analysis of the data (Table 1).

Comparison of the $K_d$ and $B_{max}$ values revealed several relationships between specific combinations of

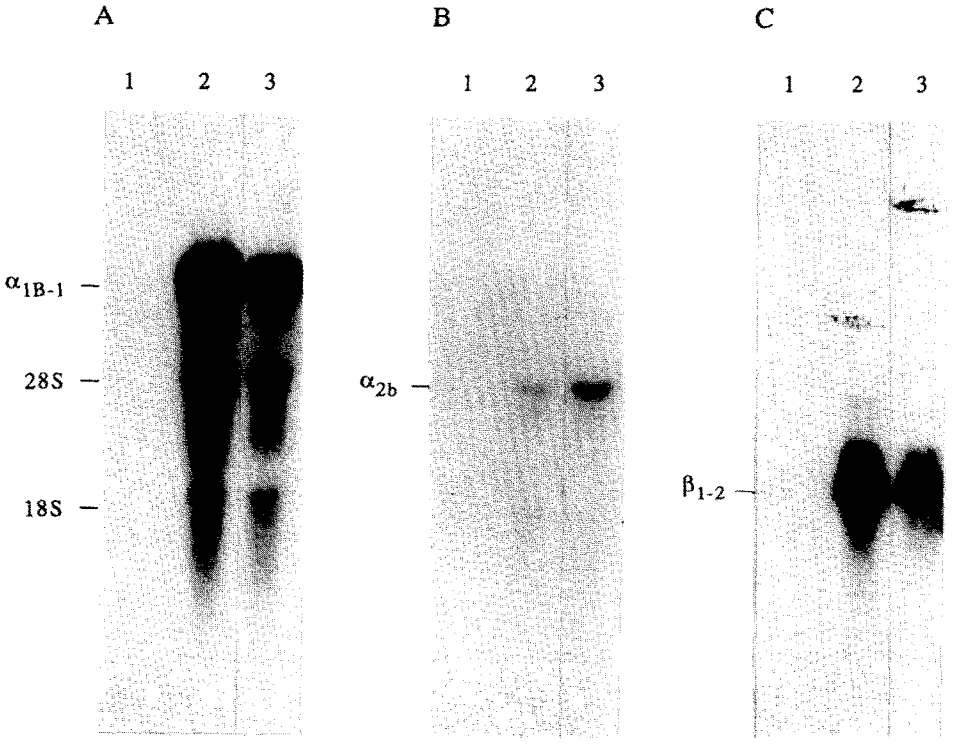


Fig. 3. Northern hybridization analysis of total mRNA from HEK293 cells transiently co-transfected with $\alpha_{1B-1}$, $\alpha_{2b}$, and $\beta_{1-2}$ human neuronal $Ca^{2+}$ channel subunit expression plasmids. Total mRNA was isolated from transfected cells 48 hr after the initiation of the transfection. 15 $\mu$g samples of total mRNA from untransfected HEK293 cells (lane 1), cells co-transfected with 8 $\mu$g pcDNA1$\alpha_{1B-1}$, 5 $\mu$g pHBCaCH$\alpha_{2b}$(A) and 2 $\mu$g pHBCaCH$\beta_{2b}$ · RBS(A) ($\beta_{1-2}$ expression plasmid) (lane 2) or co-transfected with 3 $\mu$g pcDNA1$\alpha_{1B-1}$, 11.25 $\mu$g pHBCaCH$\alpha_{2b}$ (A) and 0.75 $\mu$g pHBCaCH$\beta_{2b}$ · RBS (A) (lane 3) were electrophoresed on a formaldehyde/1% agarose gel and blotted onto a nylon support in triplicate. $\alpha_{1B-1}$, $\alpha_{2b}$ and $\beta_{1-2}$ specific mRNAs were detected by probing individual blots with their corresponding [$^{32}$P]-labeled (approximately equal specific activity) random-primed full-length cDNA probes (A, B and C, respectively). Hybridization, washing and exposure conditions were equivalent for the three blots. The location of the $Ca^{2+}$ channel subunit mRNAs ($\alpha_{1B-1}$ ~7800 nt; $\alpha_{2b}$ ~4200 nt; $\beta_{1-2}$ ~2100 nt) and 28S and 18S rRNAs are indicated in the figure.

subunits and the binding affinities and capacities of the transfected cells. In cells transfected with all three subunits, ($\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$, $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$, $\alpha_{1B\text{-}2}\alpha_{2b}\beta_{1\text{-}2}$, or $\alpha_{1B\text{-}2}\alpha_{2b}\beta_{1\text{-}3}$ transfectants) the $K_d$ values were indistinguishable ($P > 0.05$), ranging from $44.3 \pm 8.1$ to $54.9 \pm 11$ pM. In cells transfected with two-subunit combinations lacking the $\alpha_{2b}$ subunit ($\alpha_{1B\text{-}1}\beta_{1\text{-}2}$, $\alpha_{1B\text{-}1}\beta_{1\text{-}3}$, $\alpha_{1B\text{-}2}\beta_{1\text{-}2}$ or $\alpha_{1B\text{-}2}\beta_{1\text{-}3}$) the $K_d$ values were significantly lower than the three- subunit combinations ($P < 0.01$), ranging from $16.4 \pm 1.2$ to $22.2 \pm 5.8$ pM. Cells transfected with only the $\alpha_{1B\text{-}1}$ subunit had a $K_d$ value of $31.7 \pm 4.2$ pM, a value that was not different from the two-subunit combinations lacking $\alpha_{2b}$ ($P > 0.05$). However, as with the comparison between the four $\alpha_{1B}\alpha_{2b}\beta_1$ vs $\alpha_{1B}\beta_1$ combinations, when the $\alpha_{1B\text{-}1}$ was co-expressed with $\alpha_{2b}$, the $K_d$ increased significantly ($P < 0.05$) from $31.7 \pm 4.2$ to $84.6 \pm 15.3$ pM. These data demonstrate that co-expression of the $\alpha_{2b}$ subunit with $\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}1}\beta_{1\text{-}2}$, $\alpha_{1B\text{-}1}\beta_{1\text{-}3}$, $\alpha_{1B\text{-}2}\beta_{1\text{-}2}$ or $\alpha_{1B\text{-}2}\beta_{1\text{-}3}$ subunit combinations results in lower binding affinity of the cell surface receptors for [$^{125}$I]$\omega$-CgTx GVIA. This is the first evidence of the $\alpha_2$ subunit modulating the binding affinity of a cell-surface $Ca^{2+}$ channel ligand.

The $B_{max}$ values of cells transfected with various subunit combinations also differed considerably. Cells transfected with the $\alpha_{1B\text{-}1}$ subunit alone expressed a low but detectable number of binding sites (approximately 750 binding sites/cell; Table 1). When the $\alpha_{1B\text{-}1}$ subunit was co-expressed with the $\alpha_{2b}$ subunit, the binding capacity increased approximately three-fold while co-expression of a $\beta_{1\text{-}2}$ or $\beta_{1\text{-}3}$ subunit with $\alpha_{1B\text{-}1}$ resulted in 8- to 10-fold higher expression of surface binding. However, cells transfected with all three subunits expressed the highest number of cell surface receptors. The binding capacities of cells transfected with $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$ or $\alpha_{1B\text{-}2}\alpha_{2b}\beta_{1\text{-}3}$ combi-

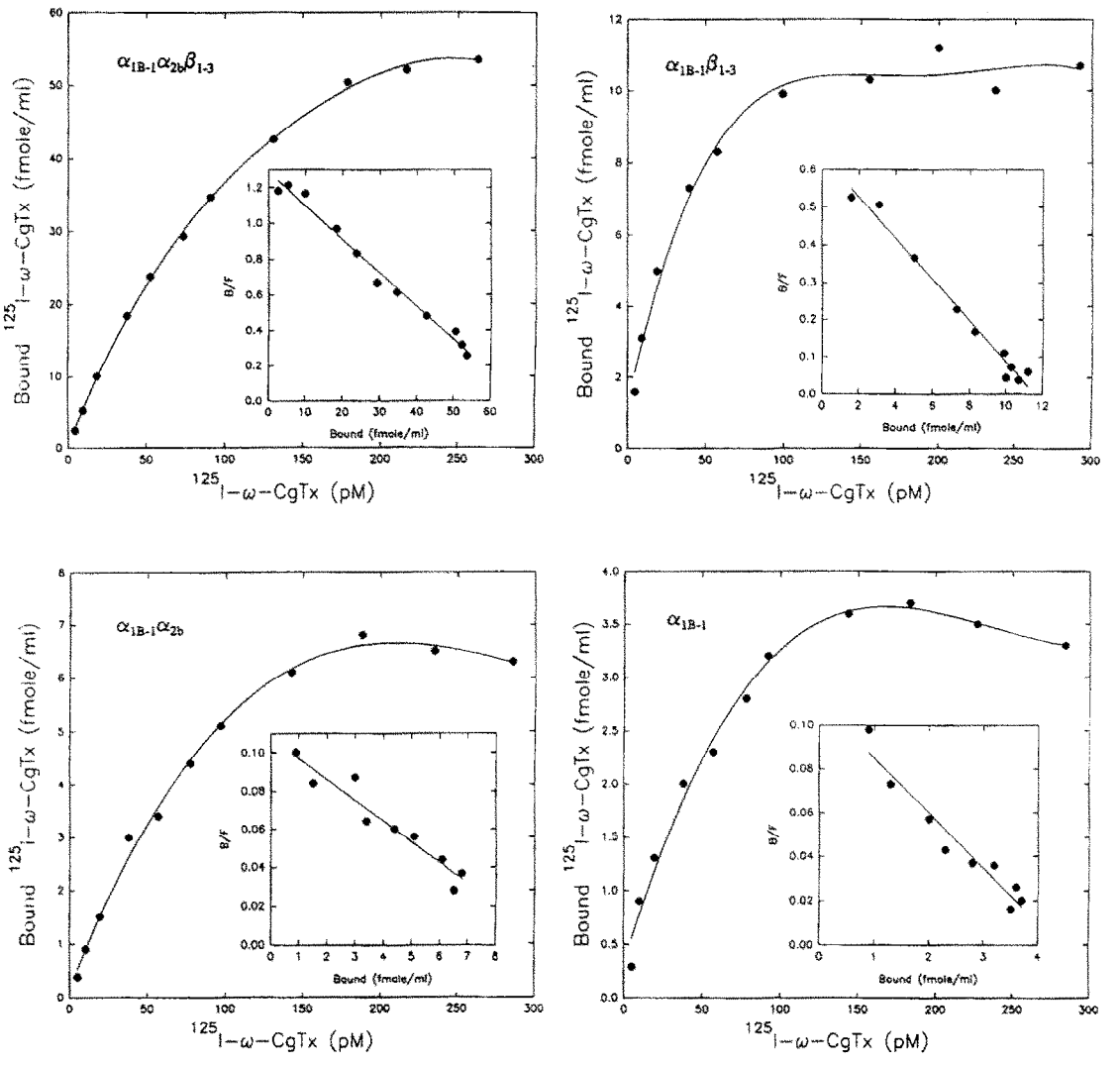


Fig. 4. Binding of [$^{125}$I]$\omega$-CgTx GVIA. HEK293 cells were co-transfected with the indicated subunit combination and assayed for specific binding of [$^{125}$I]$\omega$-CgTx GVIA as a function of increasing concentration of [$^{125}$I]$\omega$-CgTx GVIA. (Insets) Scatchard analysis of the data. B, bound; F, free.

Table 1. Summary of Scatchard analyses of [$^{125}$I]$\omega$-CgTx GVIA binding to intact cells.

| Subunit combination | $K_d$ (pM) | $B_{max}$ (sites/cell) |
|---|---|---|
| $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ | 54.9 ± 11.1 ($n$ = 4) | 45,324 ± 15,606 |
| $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$ | 53.2 ± 3.6 ($n$ = 3) | 91,004 ± 37,654 |
| $\alpha_{1B-1}\beta_{1-2}$ | 17.9 ± 1.9 ($n$ = 3) | 5756 ± 2163 |
| $\alpha_{1B-1}\beta_{1-3}$ | 17.9 ± 1.6 ($n$ = 3) | 8729 ± 2980 |
| $\alpha_{1B-1}\alpha_{2b}$ | 84.6 ± 15.3($n$ = 3) | 2256 ± 356 |
| $\alpha_{1B-1}$ | 31.7 ± 4.2 ($n$ = 3) | 757 ± 128 |
| $\alpha_{1B-2}\alpha_{2b}\beta_{1-2}$ | 53.0 ± 4.8 ($n$ = 3) | 19,371 ± 3798 |
| $\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$ | 44.3 ± 8.1 ($n$ = 3) | 37,652 ± 8129 |
| $\alpha_{1B-2}\beta_{1-2}$ | 16.4 ± 1.2 ($n$ = 3) | 2126 ± 412 |
| $\alpha_{1B-2}\beta_{1-3}$ | 22.2 ± 5.8 ($n$ = 3) | 2944 ± 1168 |
| $\alpha_{1B-2}\alpha_{2b}$ | N.D.* ($n$ = 3) | N.D.* |
| $\alpha_{1B-2}$ | N.D.* ($n$ = 3) | N.D.* |

HEK293 cells transfected with the indicated subunit cDNAs were assayed for saturation of specific [$^{125}$I]$\omega$-CgTx GVIA binding, and the data were analyzed by the Scatchard method. The $B_{max}$ determined from Scatchard analysis were corrected for transfection efficiency. 200,000 cells were used per assay except for the $\alpha_{1B-1}$, $\alpha_{1B-2}$, $\alpha_{1B-1}\alpha_{2b}$ and $\alpha_{1B-2}\alpha_{2b}$ combinations which were assayed with $1 \times 10^6$ cells per tube.

*N.D. = not detectable.

nations were approximately 2-fold higher than the corresponding combinations containing the $\beta_{1-2}$ subunit. Likewise, cells transfected with $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ or $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$ combinations expressed approximately 2.5-fold more binding sites per cell than the corresponding combinations containing $\alpha_{1B-2}$. In all cases, co-expression of the $\alpha_{2b}$ subunit with $\alpha_{1B}$ and $\beta_1$ increased the surface receptor density compared to cells transfected with only the corresponding $\alpha_{1B}$ and $\beta_1$ combinations; approximately 8-fold for $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$, 10-fold for $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$, 9-fold for $\alpha_{1B-2}\alpha_{2b}\beta_{1-2}$ and 13-fold for $\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$. Thus, in summary, the comparison of the $B_{max}$ values suggests that the toxin-binding subunit, $\alpha_{1B-1}$ or $\alpha_{1B-2}$, is more efficiently expressed and assembled on the cell surface when co-expressed with either the $\alpha_{2b}$ or the $\beta_{1-2}$ or $\beta_{1-3}$ subunit, and most efficiently expressed when both $\alpha_{2b}$ and $\beta_1$ subunits are present.

*Electrophysiological properties*

We next examined the macroscopic electrophysiological properties of $Ba^{2+}$ currents in cells transfected with various amounts of the $\alpha_{2b}$ expression plasmid while the relative amounts of $\alpha_{1B-1}$ and $\beta_{1-2}$ plasmids were held constant. The amplitudes and densities of the $Ba^{2+}$ currents (15 mM $BaCl_2$) recorded from whole cells of these transfectants differed dramatically. The average currents from 7 to 11 cells of three types of transfections (no $\alpha_{2b}$; 2:1.8:1 [$\alpha_{1B-1}$:$\alpha_{2b}$:$\beta_{1-2}$] molar ratio; and 2:10.9:1 [$\alpha_{1B-1}$:$\alpha_{2b}$:$\beta_{1-2}$] molar ratio) are shown superimposed in Fig. 5(A). The smallest currents (range: 10–205 pA) were recorded when $\alpha_{2b}$ was not included in the transfection, and the largest currents (range: 50–8300 pA) were recorded with the 2:10.9:1 ratio of $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ plasmids, the ratio that resulted in near equivalent mRNA levels for each subunit transcript. Previously we had shown that the average peak current for the $\alpha_{1B-1}\beta_{1-2}$ combination was 91 pA, whereas the 2:1.8:1 ($\alpha_{1B-1}$:$\alpha_{2b}$:$\beta_{1-2}$) ratio produced average peak currents of 433 pA. The corresponding current densities were 5 and 26 pA/pF, respectively (Williams *et al.*, 1992a). When the amount of $\alpha_{2b}$ plasmid was adjusted to yield approximately an equal abundance of subunit mRNAs [lanes (3); Fig. 3], the average peak $Ba^{2+}$ current increased from 433 to 1824 pA (4.2-fold) with a corresponding increase in average current density from 26 to 127 pA/pF (4.9-fold). This increase is in the presence of a 2.7-fold *decrease* in the mass of $\alpha_{1B-1}$ and $\beta_{1-2}$ expression plasmids in the transfections. In all transfections, the magnitudes of the $Ba^{2+}$ currents did not follow a normal distribution.

Effects on current magnitude have been observed previously when the expression of the $\beta$ subunit was varied (Hullin *et al.*, 1992). Expression of the $\beta$ subunit also has been shown to alter the peak of the current-voltage relation as well as the kinetics (activation and inactivation) of the $Ba^{2+}$ current (Wei, Perez-Reyes, Lacerda, Schuster, Brown and Birnbaumer, 1991; Castellano *et al.*, 1993a). To compare the subunit combinations and determine the effects of $\alpha_{2b}$, we examined the current-voltage properties of cells transfected with $\alpha_{1B-1}\beta_{1-2}$ or with $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ in either the 2:1.8:1 ($\alpha_{1B-1}$:$\alpha_{2b}$:$\beta_{1-2}$) molar ratio or the 2:10.9:1 ($\alpha_{1B-1}$:$\alpha_{2b}$:$\beta_{1-2}$) molar ratio transfectants. The extreme examples of no $\alpha_{2b}$ and 11.25 $\mu$g $\alpha_{2b}$ (2:10.9:1 molar ratio) are shown in Fig. 5(B). There are no significant differences in the current voltage plot at test potentials between 0 mV and +40 mV ($P < 0.05$). The slight differences seen at either side of the peak region of the current voltage plot are likely due to the normalization. The very small currents observed in the $\alpha_{1B-1}\beta_{1-2}$ transfected cells have a substantially higher component of residual leak relative to the barium current that is activated by the test pulse. When the current voltage plots are normalized, this leak is a much greater component than in the $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ transfected cells and as a result, the current–voltage plot appears broader. This is the most likely explanation of the apparent differences in the current voltage plots of Fig. 5(B), especially given the fact that the current-voltage plot for the $\alpha_{1B-1}\beta_{1-2}$ transfected cells diverge on both sides of the peak. Typically, when the voltage-dependence activation is shifted, the entire current–voltage plot is shifted, which was not observed. To qualitatively compare the kinetics of each, the average responses [Fig. 5(A)] of test pulses from −90 mV to 10 mV were normalized and superimposed [Fig. 5(C)]. No significant differences in activation or inactivation kinetics of whole-cell $Ba^{2+}$ currents were observed with any combination. These results are in contrast to those of Singer *et al.* (1991) who reported that co-injection of $\alpha_{2a}$ alters the kinetics of the $Ba^{2+}$ currents. However, endogenous *Xenopus* oocyte $Ca^{2+}$ channel activity (Dascal *et al.*, 1986; Singer-Lahat *et al.*, 1992; Williams *et al.*, 1992b) may have affected their results.

## DISCUSSION

One $\alpha_2$ (Ellis, Williams, Ways, Brenner, Sharp, Leung, Campbell, McKenna, Koch, Hui, Schwartz and Harpold, 1988) and four $\beta$ (Ruth *et al.*, 1989; Hullin *et al.*, 1992; Perez-Reyes *et al.*, 1992; Castellano *et al.*, 1993b) $Ca^{2+}$ channel subunit genes have been identified. Each of the genes is expressed in neuronal tissue, although transcripts also have been observed in other tissues (see Snutch and Reiner, 1992 and Tsien *et al.*, 1991, for reviews; Seino, Chen, Seino, Blondel, Takeda, Johnson and Bell, 1992; Yaney, Wheeler, Wei, Perez-Reyes, Birnbaumer, Boyd and Moss, 1992; Yu, Hebert, Brenner and Lytton, 1992; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Hullin *et al.*, 1992). Furthermore, splice variants of $\alpha_2$, $\beta_1$ and $\beta_2$ have been identified (Williams *et al.*, 1992b; Hullin *et al.*, 1992; Pragnell *et al.*, 1991;

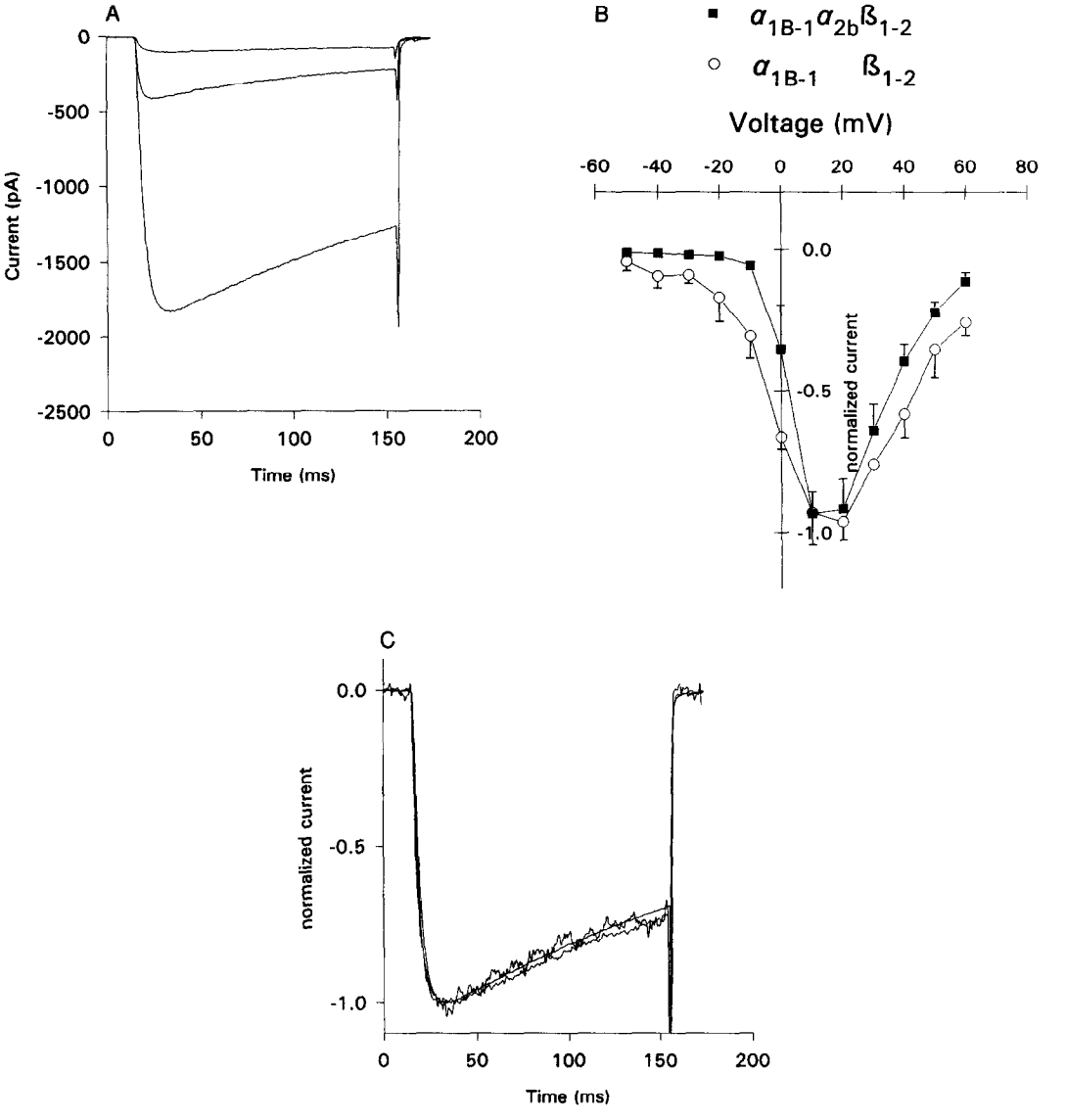


Fig. 5. Whole-cell $Ba^{2+}$ currents recorded from HEK293 cells transiently co-transfected with $\alpha_{1B-1}$, $\alpha_{2b}$ and $\beta_{1-2}$ human neuronal $Ca^{2+}$ channel subunit expression plasmids. (A) Superimposed averaged $Ba^{2+}$ currents from HEK293 cells transiently transfected with $\alpha_{1B-1}\beta_{1-2}$ (smallest current, average of 7 cells), $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ with molar ratio of 2:1.8:1 (middle record, average of 10 cells) or $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ with a molar ratio of 2:10.9:1 (largest current, average of 10 cells). $Ba^{2+}$ currents (15 mM $BaCl_2$) are from a holding potential of −90 mV to a test potential of +10 mV. (B) Current voltage plot from HEK293 cells transiently transfected with $\alpha_{1B-1}\beta_{1-2}$ or $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ (molar ratio 2:10.9:1). Current voltage plots are the mean ± SD from 5 cells. The current voltage plots from individual cells were normalized to the peak current prior to averaging. No significant difference in the current voltage relations were observed. (C) Superimposed normalized averaged $Ba^{2+}$ currents from (A). The currents were normalized to the peak current recorded during the test pulse. No significant difference in the time-course of activation or inactivation could be detected.

Perez-Reyes *et al.*, 1992; Kim *et al.*, 1992), a splice variant of $\beta_3$ has been suggested (Castellano *et al.*, 1993a) and some of these variants are expressed in a tissue-specific manner (Williams *et al.*, 1992b; Kim *et al.*, 1992; Powers *et al.*, 1992). To study human neuronal voltage-dependent $Ca^{2+}$ channels, in addition to cloning $\alpha_1$ subunits, we characterized several $\alpha_2$ and $\beta_1$ splice variants. We have identified five $\alpha_2$ splice variants ($\alpha_{2a}$ to $\alpha_{2e}$; Fig. 1). Of these, $\alpha_{2b}$ appears to be exclusively expressed in neuronal tissue (Williams *et al.*, 1992b; Kim *et al.*, 1992; Fig. 1). Five splice variants of the $\beta_1$ gene also have been identified ($\beta_{1\text{-}1}$ to $\beta_{1\text{-}5}$; Fig. 2), although $\beta_{1\text{-}4}$ and $\beta_{1\text{-}5}$ appear to be aberrant forms and may not encode functional protein. Of these, $\beta_{1\text{-}2}$ and $\beta_{1\text{-}3}$ appear to be exclusively expressed in neuronal tissue (unpublished observation).

Recombinant voltage-dependent $Ca^{2+}$ channels have been functionally characterized in *Xenopus* oocytes (Perez-Reyes *et al.*, 1992; Mikami *et al.*, 1989; Singer *et al.*, 1991; Wei *et al.*, 1991; Castellano *et al.*, 1993a; Hullin *et al.*, 1992; Mori *et al.*, 1991; Williams *et al.*, 1992b), mouse L cells (Perez-Reyes *et al.*, 1989; Kim, Wei, Ruth, Perez-Reyes, Flockerzi, Hofmann and Birnbaumer, 1990; Lacerda *et al.*, 1991; Varadi *et al.*, 1991), African Green monkey kidney COS cells (Perez-Reyes *et al.*, 1992), Chinese hamster ovary (CHO) cells (Bosse *et al.*, 1992) and HEK293 cells (Williams *et al.*, 1992a). In the absence of suitable immunological reagents, none of these studies carefully examined the contribution to the cell surface expression of each subunit of the multimeric recombinant voltage-dependent $Ca^{2+}$ channels. To address this issue, we have used the transient expression of $Ca^{2+}$ channel subunits in HEK293 cells, a cell line that has no significant endogenous $Ca^{2+}$ channel activity, and the high-affinity binding of $\omega$-CgTx GVIA to $\alpha_{1B}$-mediated $Ca^{2+}$ channel complexes to monitor the cell surface expression of the receptors.

We used the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ subunit combination to analyze the effects of varying the expression levels of subunit transcripts on $\omega$-CgTx GVIA binding. Our initial report of functional $\alpha_{1B}$-mediated $Ca^{2+}$ channels showed subunit combination-dependent differences in $\omega$-CgTx GVIA cell surface expression and current density measurements of the $Ba^{2+}$ current (Williams *et al.*, 1992a). Because the $\alpha_{2b}$ transcript in the transfected cells was 5- to 10-fold less abundant than the $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ transcripts [lanes (2), Fig. 3], it is possible that the $Ca^{2+}$ channel complexes expressed on the cell surface might have represented a heterogeneous population of combinations of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ subunits in these studies. The reason for the relative low steady-state $\alpha_{2b}$ mRNA level in cells co-transfected with the 2:1.8:1 molar ratio of expression plasmids is unknown. Transcription of each of the subunit cDNAs is controlled by the cytomegalovirus (CMV) promoter and, thus, there might be competition for transcription factors of which the $\alpha_{2b}$ construct could be the least efficient. Alternatively, the $\alpha_{2b}$ transcript might be less stable than the $\alpha_{1B\text{-}1}$ or $\beta_{1\text{-}2}$ transcripts. In the present study, however, we altered the relative molar ratio of $\alpha_{2b}$ expression plasmid in the transfections (6-fold increase) and achieved approximately equal abundance of the $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ transcripts [lanes (3), Fig. 3].

The increase in molar quantity of $\alpha_{2b}$ expression plasmid relative to $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ expression plasmids resulted in an 8-fold increase in the $\omega$-CgTx GVIA sites per cell of the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ combination compared to the $\alpha_{1B\text{-}1}\beta_{1\text{-}2}$ combination (Table 1). This represents a significant increase over the 2.4-fold difference in these two combinations that we previously observed (Williams *et al.*, 1992a). Furthermore, a 1.6-fold increase in the average receptor sites per cell was observed even with a 2.7-fold decrease in the *mass* of $\alpha_{1B\text{-}1}$ plasmid that was transfected in the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ combination (8 $\mu$g compared to 3 $\mu$g; Williams *et al.*, 1992a; Table 1).

In the present study using HEK293 cells for transient expression, the 6-fold molar increase of the $\alpha_{2b}$ expression plasmid, relative to $\alpha_{1B\text{-}1}$ and $\beta_{1\text{-}2}$ expression plasmids, also resulted in a 4.9-fold increase in the $Ba^{2+}$ current density over our previous determination (Williams *et al.*, 1992a). Comparison of the current densities determined in cells co-transfected with the optimal ratio of $\alpha_{1B\text{-}1}$, $\alpha_{2b}$ and $\beta_{1\text{-}2}$ expression plasmids (2:10.9:1) to cells co-transfected with the 2:1.8:1 molar ratio revealed that an increased number of *functional* $Ca^{2+}$ channel complexes was expressed on the cell surface consistent with the increased number of toxin binding sites. Interestingly, whereas the $\omega$-CgTx GVIA binding sites were increased $\sim$1.6-fold (compare 45,324 sites/cell, Table 1 to 28,710 sites/cell, Williams *et al.*, 1992a), the current density was increased $\sim$5-fold (127 pA/pF, this work; 26 pA/pF, Williams *et al.*, 1992a). Furthermore, the average current density with the optimal ratio (127 pA/pF) is $\sim$25-fold higher than the average current density of cells co-transfected with the $\alpha_{1B\text{-}1}\beta_{1\text{-}2}$ expression plasmid combination. These results further support a significant role for the $\alpha_{2b}$ subunit in facilitating the assembly of the $Ca^{2+}$ channel complex in the plasma membrane that results in an increase in the number of functional channels on the cell surface. While it appears that the $\alpha_{2b}$ subunit also plays a functional role in modulating $\omega$-CgTx GVIA affinity (see below), we have not observed any effect on the kinetics of activation and inactivation of the whole cell $Ba^{2+}$ currents or the current–voltage properties of the $Ca^{2+}$ channel complex. In addition, the relatively greater increase in current density compared to $\omega$-CgTx GVIA binding sites, observed in this study, suggests that the $\alpha_{2b}$ subunit may also affect $Ca^{2+}$ channel function by causing a change in certain microscopic channel properties such as an increase in single channel conductance. Additional studies are required to resolve this issue.

Recombinant expression of the $\alpha_{1B\text{-}1}$ subunit of the

human $\alpha_{1B}$-mediated $\omega$-CgTx GVIA-sensitive voltage-dependent $Ca^{2+}$ channels in HEK293 cells results in the expression of a single class of high-affinity [$^{125}$I]$\omega$-CgTx GVIA binding sites with a $K_d$ of $31.7 \pm 4.2$ pM and a binding capacity of $757 \pm 128$ receptors per cell. The expression of the toxin binding subunit on the cell surface is markedly increased when both a $\beta_1$ subunit and the $\alpha_{2b}$ subunits are co-expressed with an $\alpha_{1B}$ subunit. The three-subunit combinations result in an increase in receptor density of approximately 60-fold for the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}2}$ and 120-fold for the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$ combinations (Table 1). Cells co-transfected with either an $\alpha_{1B}\alpha_{2b}$ or $\alpha_{1B}\beta_1$ combination express low levels of $\omega$-CgTx GVIA binding sites compared to the trimeric transfectants.

The presence of the $\alpha_{2b}$ subunit in the receptor complex significantly increases the $K_d$ for $\omega$-CgTx GVIA, whereas, the presence of a $\beta_1$ subunit appears to have less of an effect, perhaps slightly decreasing the $K_d$. Nevertheless, in all cases, the $K_d$ values are sufficiently low to be considered high-affinity. Based on related studies, others (Varadi *et al.*, 1991; Lacerda *et al.*, 1991) have reported that recombinant co-expression of the skeletal muscle $\alpha_{1skm}$ subunit with the $\beta_{1\text{-}1}$ subunit in L cells results in a three-to-ten-fold increase in binding capacity without any change in affinity of DHP binding compared to cells transfected with $\alpha_1$ alone. Taken together, our results of the $\omega$-CgTx GVIA binding experiments indicate that neither the $\alpha_{2b}$ subunit nor a $\beta_1$ subunit is solely responsible for the increased expression of $\alpha_{1B}$ toxin binding site at the cell surface and that all three subunits contribute in detectable and significant ways to the efficient assembly of the multimeric, $\omega$-CgTx GVIA-sensitive voltage-dependent $Ca^{2+}$ channel receptor complex.

The results of the present study clearly establish that co-expression of $\alpha_{1B}$, $\alpha_{2b}$ and $\beta_1$ is required for efficient expression and assembly of the $Ca^{2+}$ channel complex on the cell surface. Previously, several plasma membrane multimeric complexes have been shown to require translation and assembly in the endoplasmic reticulum (ER) for efficient transport to the cell surface. For example, the T-cell antigen receptor (TCR) is a seven subunit-membrane complex of which several of the subunits are expressed in excess and rapidly degraded, whereas, the complete complex is quite stable (Minami, Weissman, Samelson and Klausner, 1987). Similarly, the muscle-type nicotinic acetylcholine receptor (nAchR) is a five-subunit ($\alpha_2\beta\gamma\delta$) membrane complex and the complex is assembled in a stepwise mechanism (Gu, Forsayeth, Verrall, Yu and Hall, 1991). Furthermore, when nAchR $\alpha\gamma$ or $\alpha\delta$ hetero-dimers are co-expressed in the absence of other subunits, less than 0.5% of the nAchR (measured as $\alpha$-bungarotoxin binding sites) are assembled on the cell surface. Whereas, in BC3H-1 cells, a cell line that expresses $\alpha_2\beta\gamma\delta$ muscle-type nAchRs, approximately 65% of the binding sites were found on the cell surface (Boulter and Patrick, 1977). Our results suggest that surface expression of an $\alpha_{1B}\alpha_{2b}\beta_1$ $Ca^{2+}$ channel complex also might be regulated by co-translation and assembly of each subunit in the ER. Once appropriate immunological reagents are available, the kinetics of synthesis and transport of each subunit and the associated $Ca^{2+}$ channel complex to the cell surface can be characterized.

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