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Acidic motif responsible for plasma membrane association of the voltage-dependent calcium channel β1b subunit

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Abstract

Voltage-dependent calcium channels consist of a pore-forming transmembrane α1-subunit, which is known to associate with a number of accessory subunits, including α2-δ- and β-subunits. The β-subunits, of which four have been identified (β1–4), are intracellular proteins that have marked effects on calcium channel trafficking and function. In a previous study, we observed that the β1b-subunit showed selective plasma membrane association when expressed alone in COS7 cells, whereas β3 and β4 did not. In this study, we have examined the basis for this, and have identified, by making chimeric β-subunits, that the C-terminal region, which shows most diversity between β-subunits, of β1b is responsible for its plasma membrane association. Furthermore we have identified, by deletion mutations, an 11-amino acid motif present in the C terminus of β1b but not in β3 (amino acids 547–556 of β1b, WEEEEDYEEE), which when deleted, reduces membrane association of β1b. Future research aims to identify what is binding to this sequence in β1b to promote membrane association of this calcium channel subunit. It is possible that such membrane association is important for the selective localization or clustering of particular calcium channels with which β1b is associated.

Introduction

The α1 subunit of voltage-dependent calcium channels (VDCCs) is a transmembrane protein which forms the ion-conducting pore and contains the voltage-sensor of the channel. To date, 10 different α1-subunits have been identified by molecular cloning; i.e. α1A-I and α1S (Perez-Reyes & Schneider, 1994; Birnbaumer *et al*., 1994; Perez-Reyes *et al*., 1998; Cribbs *et al*., 1998). A number of accessory subunits, including α2-δ- and β-subunits, are known to associate with α1-subunits. The α2-subunit is a large extracellular protein linked by a disulphide bridge to a smaller, transmembrane δ-protein, derived from the same gene. A number of β-subunit isoforms have been cloned (β1–4), some of which possess alternatively spliced variants (Ruth *et al*., 1989; Perez-Reyes & Schneider, 1994). The β-subunits are hydrophilic, intracellular proteins, which were first shown to interact with α1-subunits via a highly conserved binding sequence located on the intracellular loop between domains I and II of the α1-subunit (α-interaction domain, AID, Pragnell *et al*., 1994). The corresponding region of interaction in the β-subunit is even more highly conserved between β-subtypes (β-interaction domain, BID, Pragnell *et al*., 1994), suggesting that the α1–β interaction is of great importance to the action of these proteins *in situ*. More recently, β-subunits have also been found to associate with both the N and C terminus of particular α1-subunits (Birnbaumer *et al*., 1998), and it is possible that all three elements form part of a complex β-subunit-binding pocket (Walker *et al*., 1999; Canti *et al*., 1999).

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All β-subunits contain two major conserved regions, with the BID at the beginning of the second conserved region. We have recently shown in a structural modelling study that the β-subunit, β1b, contains three distinct domains that have been shown to be involved in protein–protein interactions in many other systems (Hanlon *et al*., 1999). Amino acids 1–100 form a putative PDZ domain, amino acids 100–161 in the first conserved domain form a *src* homology (SH)-3 domain; and amino acids 218–430, in the second conserved region, form a guanylate kinase (GK) domain. The BID motif is associated with the start of the GK domain (Hanlon *et al*., 1999). Such a domain signature of $(PDZ)_{1-3}$–SH3–GK is a hallmark of a growing family of membrane-associated guanylate kinase (MAGUK) proteins, involved, e.g. in membrane protein anchoring (Sheng & Wyszynski, 1997).

Native N, L and P/Q type VDCCs are thought to exist as hetero-oligomers, consisting minimally of an α1-subunit and accessory α2-δ- and β-subunits. Of those that have been purified, the skeletal muscle calcium channel was found to consist of α1S, β1a, α2-δ and γ (Takahashi *et al*., 1987). The N-type channel, purified by virtue of binding ω-conotoxin GVIA, consists of α1B-, α2-δ- and one of several different β-subunits, particularly β3 or β1b (Witcher *et al*., 1993; Vance *et al*., 1998). No γ-subunit was found to co-purify with this complex. The P/Q-type channel was found to consist of α1A-, α2-δ- and any of the four neuronal β-subunits (β1b, β2, β3 and β4, Liu *et al*., 1996). The *in vitro* affinity of the α1A AID for the different β-subunits showed the order β4 > β2a > β1b >> β3 (De Waard *et al*., 1995). These and other results show clearly that different β-subunits are associated to varying extents with different α1-subunit complexes, although it remains unclear whether this is primarily because of varying affinity or because of differential expression in different cell types..

There are a number of functional consequences of interaction between these calcium channel subunits, which have been summarized in a recent review (Birnbaumer *et al*., 1998). VDCC β-subunit

co-expression has been shown to increase the number of 1,4-dihydropyridine-binding sites (Lacerda *et al*., 1991), and also to enhance macroscopic current (Berrow *et al*., 1997; Stephens *et al*., 1997), which has been attributed to increased expression of channel protein at the plasma membrane (Brice *et al*., 1997; Gao *et al*., 1999; Brice & Dolphin, 1999), and to changes in channel biophysical properties including increased coupling between movement of the voltage-sensors and channel opening (Neely *et al*., 1993; Jones *et al*., 1998), as well as an increase in mean open time (Costantin *et al*., 1998). Much of the early work was performed by over-expression of the subunits in *Xenopus* oocytes, but there is a recent report that this expression system contains an endogenous calcium channel β-subunit ($\beta3_{Xo}$, Tareilus *et al*., 1997). We have found in COS-7 cells that all heterologously expressed β-subunits traffic α1A-subunits to the plasma membrane (Brice *et al*., 1997), and others have found similar results in HEK293 cells for α1C and β2a (Chien *et al*., 1995). In reciprocal experiments, in which native β-subunits were depleted by injection of an antisense oligonucleotide, calcium channel currents were markedly attenuated in DRG neurons (Berrow *et al*., 1995).

One mechanism whereby β-subunits produce trafficking of α1-subunits was proposed to be that they themselves are targeted to the plasma membrane. Plasma membrane association has been observed for β1b (Brice *et al*., 1997) and β2a (Chien *et al*., 1995; Brice *et al*., 1997). Rat, rabbit and human β2a-subunits are palmitoylated at the N terminus (for review see Birnbaumer *et al*., 1998), but a palmitoylation-deficient mutant is still able to traffic α1C to the plasma membrane. However, when this β2a mutant is expressed alone, although it is still membrane-associated, it shows a modified subcellular distribution, with little at the plasma membrane (Chien *et al*., 1996, 1998). Furthermore, we have observed that while the other β-subunits, β3 and β4, are not themselves associated with the plasma membrane, all β-subunits are able to traffic α1-subunits to the plasma membrane in COS-7 cells (Brice *et al*., 1997).

In the present study we wished to examine the mechanism by which β1b, which is not palmitoylated, associates with the plasma membrane in COS-7 cells. We have made chimeras between β1b and β3 by exchanging their C-terminal non-conserved regions. We have also made a number of β1b truncation and deletion constructs, to identify the motif(s) involved in plasma membrane association.

Materials and methods

Materials

Calcium channel cDNAs were obtained from the following sources. Rat α1A (M64373), rat α1C (M67515) and β1b (Tomlinson *et al*., 1993) were obtained from Dr T. Snutch (UBC, Vancouver, Canada), rabbit α1B (D14157) was obtained from Dr Y. Mori (Okazaki, Japan), rat β2a (M80545), β3 (M88751) and β4 (M80545) cDNAs were obtained from Dr E. Perez-Reyes (Loyola University, IL, USA). Rat α2-δ (M86621) was obtained from Dr H. Chin (NIH, Bethesda MD, USA).

Molecular biology

Construction of β1b/β3 and β3/β1b chimeras

The β1b-subunit used in this study is that of Tomlinson *et al*. (1993). It is identical to the rat β1b clone in the database (X61394) except for two substitutions (R417S and V435A) and a deletion of A431 (T. Snutch, personal communication). The numbering given in this paper refers to the β1b clone that we have used.

The C-termini of β1b and β3 were swapped from within a region conserved between the proteins. An *Mfe*I site is found at nucleotide position 1241 of the β1b cDNA (X61394). PCR was used to introduce an *Mfe*I site into position 1115 of β3 (M88751) by changing CAGCTG to CAATTG. This does not change the amino acid sequence. Swapping the C-termini at the *Mfe*I sites produces chimeras consisting of amino acids 1–393 β1b, 342–484 β3 (β1b/3) and 1–341 β3, 394–596 β1b (β3/1b). The sequences of the constructs were verified by cycle sequencing using the SequiTherm Excel II kit (Epicentre Technologies, Madison, USA).

Production of β1b truncations and deletions

Truncation constructs of β1b, named β1bΔ570–596 and β1bΔ547–596 were made by PCR . The forward primer for both constructs was as follows: 5′-TCT CTG GTA CCG GAC CGG AGG ATC CTC. The sequence of the reverse primer for the β1bΔ570–596 construct was as follows: 5′-ACA TTA GCG GGC CTT ATT C. The sequence of the reverse primer for the β1bΔ547–596 construct was as follows: 5′-TCA GGA GCC CTG CCG AGC. Following the PCR, the products were subcloned into pMT2 vector and used for the transfection experiments. The sequences of the primer pair for the deletion construct β1bΔ547–556 were as follows: 5′-GGA GCC CTG CCG AGC TG and 5′-ATG ACC GAC AAC AGG AAC. PCR was performed on the β1b template subcloned into the pMT2 vector. The PCR product was purified by agarose gel electrophoresis, digested for 1 h by *Dpn* I restriction endonuclease, religated and transformed into *Escherichia coli* XL-1 Blue cells (Stratagene, La Jolla, USA). The sequences of all truncation and deletion constructs of β1b were verified by sequencing using Sequenase V. 2.0 kit (Amersham-Pharmacia, UK).

Cell culture and transfection

COS-7 cells

COS-7 cells were cultured and transfected by electroporation as previously described (Campbell *et al*., 1995; Brice *et al*., 1997). The β subunit cDNAs in the vector pMT2 were used at 5 μg per transfection. Following transfection, cells were maintained at 37 °C for ~48 h, and then replated using non-enzymatic cell dissociation medium (Sigma), and maintained at 25 °C for between 1 and 2 h prior to immunocytochemistry.

Madin Darby canine kidney (MDCK) cells

MDCK cells were cultured and microinjected as described previously (Brice & Dolphin, 1999). They were cultured in Dulbecco's minimum essential medium (DMEM), containing 10% foetal calf serum, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, at 37 °C. For experiments, MDCK cells were grown on Costar transwell filters which allow the cells to become fully polarized. Cells were plated onto the filters at 10^6 cells per well, and the medium was changed every 2–3 days. After 10–17 days on the filters, the cells were used for microinjection, only if they were fully confluent and the monolayer intact when examined under magnification of × 400. The nuclei of individual cells were micro-injected with the stated cDNAs (0.1 μg μL^{-1}) using an Eppendorf microinjector (0.1 s, 140 hPa), within an area marked on the filter, so that it could be located for subsequent visualization. Cells were then returned to the incubator. Each subunit combination was micro-injected into at least three sets of polarized MDCK cells.

Immunocytochemistry

COS-7 cells were fixed 48 h after transfection. MDCK cells were fixed 6 h after microinjection. The cells were washed twice in Tris-buffered saline (TBS; 154 mM NaCl, 20 mM Tris, pH 7.4), then fixed in 4% paraformaldehyde in TBS as described (Brice *et al*., 1997). The

cells were permeabilized in 0.02% Triton X-100 in TBS and incubated with blocking solution [20% (v/v) goat serum, 4% (w/v) bovine serum albumin (BSA), 0.1% (w/v) D,L-lysine in TBS]. The cells were incubated for 14 h at 4 °C with the appropriate primary antibody diluted in 10% goat serum, 2% BSA, 0.05% D,L-lysine. The VDCC antibodies used in this study were either raised in rabbits against specific peptides derived from a sequence common to all β-subunits (β_{common}, Campbell *et al.*, 1995), used at 1 : 500 dilution, or were produced as monoclonal antibodies to fusion proteins derived from the C-terminal sequences of β1b or β3, used at 5 μg mL^{-1}. The β1b and β3 fusion proteins consisted of amino acids 453–646 of human β1b, and 403–525 of human β3. The antibodies recognize the rat orthologues, but do not cross-react with other β-subunits (Day *et al.*, 1998).

The primary polyclonal antibodies were detected using biotin-conjugated goat anti-rabbit IgG (0.5 μg mL^{-1}, Sigma), then streptavidin FITC (15 μg mL^{-1}) or Texas Red (5 μg mL^{-1}, Molecular Probes, Eugene, OR, USA). For the primary monoclonal antibodies, detection was with goat anti-mouse FITC or Texas Red (both 10 μg mL^{-1}, Molecular Probes). In some experiments in COS-7 cells, cells were then incubated for 20 min with the double-stranded DNA dye YO-YO (2.4 nM, Molecular Probes) to visualize the nucleus. Cells were then washed in TBS for 5 × 5 min. For the COS-7 cells, coverslips were mounted directly onto a microscope slide with vectorshield (Vector Laboratories, CA, USA), whereas for the MDCK cells, filters were mounted between a coverslip and a microscope slide. Cells were examined either on an laser scanning confocal microscope (either MRC 1024, Bio-Rad, Hemel Hempstead, UK or Leica TCS SP, Milton Keynes, UK), using conditions of constant aperture and gain, ensuring that the image was not saturated. The optical sections are 1 μm. For MDCK cells, confocal images are shown in the X–Y plane and in the X–Z plane.

Expression of constructs and electrophysiological recording in Xenopus *oocytes*

Adult female *Xenopus laevis* were killed by anaesthetic overdose, in a 0.25% solution of tricaine, decapitated and pithed. Oocytes were removed and defolliculated by treatment with 2 mg/mL collagenase type Ia in a Ca^{2+}-free ND96 saline [containing (in mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; HEPES, 5, pH adjusted to 7.4 with NaOH] for 2 h at 21 °C. Plasmid cDNAs for the α1B calcium channel, plus accessory α2δ and either β1b or β3, or the chimeric and truncated β-subunits, and rat D2 dopamine receptors, were mixed in a ratio of 3 : 1 : 4 : 3, and ~ 10 nL was injected into the nuclei of stage V or VI oocytes. Injected oocytes were incubated at 18 °C for 3–7 days in ND96 saline (as above plus 1.8 mM $CaCl_2$) supplemented with 100 μg/mL penicillin, 100 IU/mL streptomycin (Gibco) and 2.5 mM Na pyruvate. Whole-cell recordings from oocytes were made in the two-electrode voltage-clamp configuration with a chloride-free solution containing (in mM): $Ba(OH)_2$, 5; TEA-OH, 80; NaOH, 25; CsOH, 2; HEPES, 5 (pH 7.4 with methanesulphonic acid). In all experiments oocytes were injected with 30–40 nL of a 100 mM solution of K_3-1,2-bis (aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), 1 h before recording, in order to suppress endogenous Ca^{2+}-activated Cl^- currents. Electrodes contained 3 M KCl, and had resistances of 0.3–2 MΩ. The holding potential (V_H) was –100 mV. All illustrated traces are at a test potential of 0 mV, and the current amplitude was always measured 20 ms after the start of the test pulse. Membrane currents were recorded every 15 s, amplified and low-pass filtered at 1 kHz using a Geneclamp 500 amplifier and digitized through a Digidata 1200 interface (Axon Instruments, CA, USA). In all cases currents were leak subtracted on-line by a P/4 protocol. Current–voltage relationships were fitted with a modified Boltzmann equation $I = G_{max} \times (V - V_{rev})/\{1 + \exp[-(V - V_{50,act})/k]\}$, where G_{max} is the maximum conductance; V_{rev} is the reversal potential; k is the slope factor and $V_{50,act}$ is the voltage for 50% current activation.

Results

VDCC β-subunit distribution in COS-7 and MDCK cells

Heterologous expression of β1b and β2a in COS-7 cells resulted in immunolocalization associated with the plasma membrane (Fig. 1A and B, left panel). To be certain that the membrane association was not an artefact resulting from the presence of a large nucleus directly apposed to the plasma membrane, in some experiments we counter-stained the nucleus with the DNA dye YO-YO (Fig. 1A, right panel). In contrast, β3 and β4 showed a cytoplasmic localization in all experiments (Fig. 1C and D, left panel). For β1b and β3, similar results were obtained using the polyclonal β_{common} antibody (used in Fig. 1) and either β1b or β3 monoclonal antibodies (results not shown). No immunostaining was observed in untransfected COS-7 cells with these antibodies (results not shown).

We have previously used the polarized MDCK cell line to investigate the trafficking of VDCC α1 subunits (Brice & Dolphin, 1999). Using the same technique of microinjection into the nuclei of polarized cells, we have now observed that when the β-subunits are

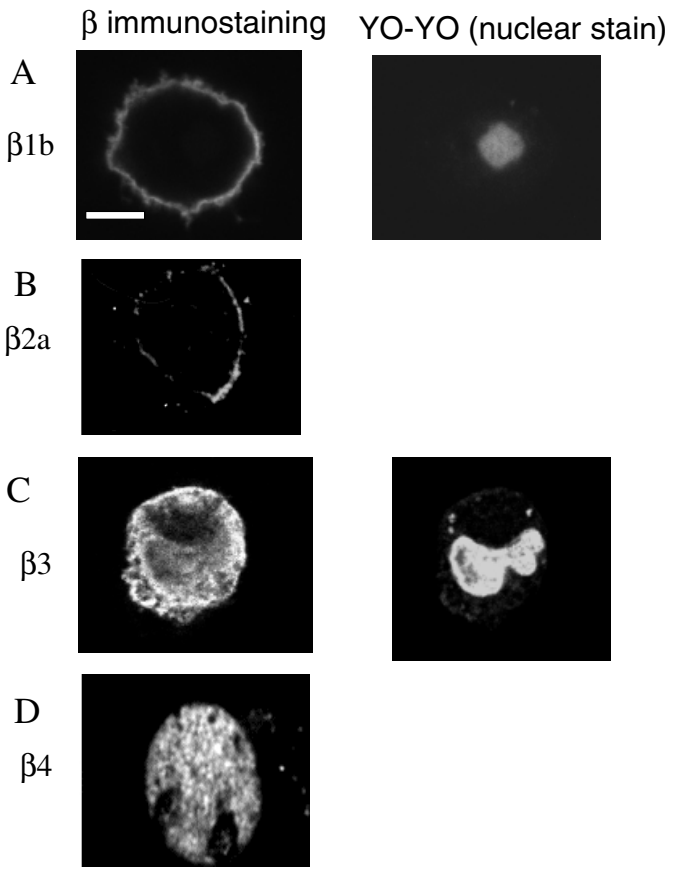


FIG. 1. Localization of β-subunits when heterologously expressed alone in COS-7 cells. Left panel: immunolocalization of (A) β1b, (B) β2a, (C) β3 and (D) β4 in horizontal sections through COS-7 cells transfected with these subunits individually, stained with the β_{common} antibody. Right panel: for β1b and β3, the nuclear stain YO-YO was also used. These results are representative of 17, eight, seven, eight different transfections, respectively, with five to eight cells examined per experiment. Calibration bar, 10 μm.

expressed alone, the β1b- and β2a-subunits are clearly associated with the basolateral membrane (Fig. 2A and B), as to a lesser extent was β4, although it was generally cytoplasmic, whereas β3 showed no association with either the basolateral or apical membranes, the staining being largely cytoplasmic (Fig. 2C and D). The data have been quantified in Table 1. When β1b was co-expressed with α1A and α2-δ its localization became apical (Table 1), the same localization as α1A in MDCK cells in this combination of subunits (Brice & Dolphin, 1999). In contrast, in combination with α1C and α2-δ, the distribution of β1b remained primarily basolateral, as we have found previously for α1C expressed with α2δ- and any β-subunit (Brice & Dolphin, 1999).

In subsequent experiments, we have compared the membrane-associated β1b with the cytoplasmically localized β3, to examine the basis for the differences in their localization.

Effect of inhibition of protein synthesis on the distribution of β1b- and β3-subunits in COS-7 cells

We have previously shown that although β3 is not associated with the plasma membrane, like the other β-subunits it is found to pellet with a membrane fraction on differential centrifugation (Campbell *et al*., 1995). Thus, it is likely that β3 is associated with the endoplasmic reticulum, where it may represent a pool of protein that is misfolded in the absence of an α1-subunit. Under these conditions it should be rapidly degraded by proteolysis, and we have thus compared the turnover of β1b and β3. The protein synthesis inhibitor cycloheximide (50 μM) was applied to the COS-7 cells 2, 4 or 6 h before fixation, and the effect examined on the distribution of the two β-subunits. The immunostaining for β3 was almost completely lost from the cells at all time points (see Fig. 3A for the effect of 2 h cycloheximide), suggesting that it represents a pool of protein that is rapidly turned over. Furthermore, the immunostaining for β3 after cycloheximide treatment did not reveal any residual plasma membrane-associated β3 (Fig. 3A). In contrast, the immunolocalization of β1b was not affected by cycloheximide treatment for 2–6 h suggesting that it is correctly folded and possibly stabilized by association with a target in the membrane (Fig. 3B).

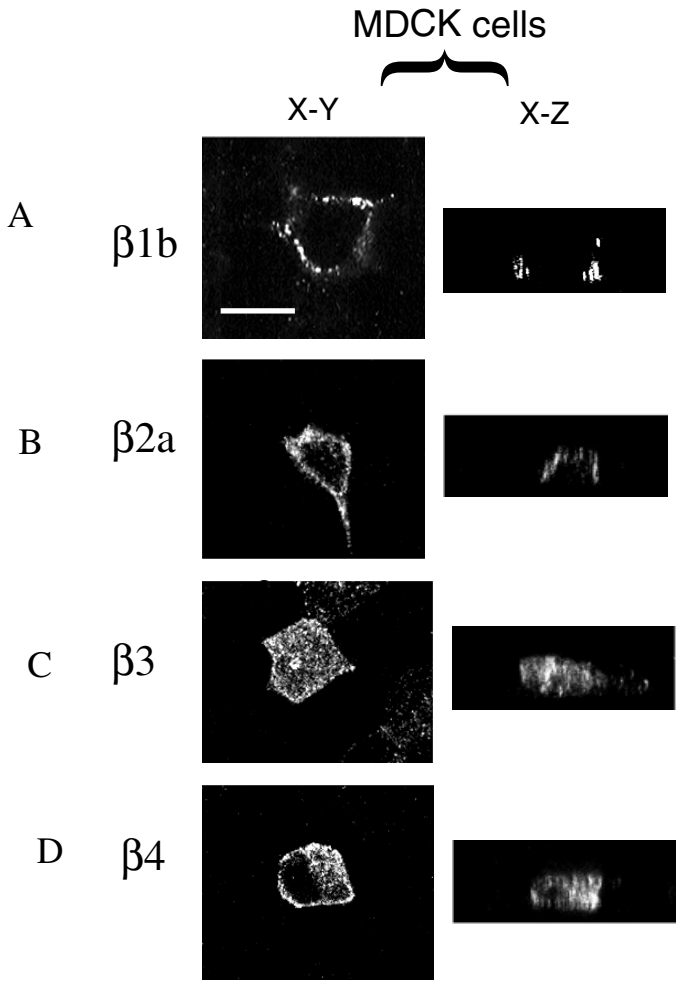


FIG. 2. Localization of β-subunits when heterologously expressed alone in MDCK cells. Left panel: immunolocalization of (A) β1b, (B) β2a, (C) β3 and (D) β4 in horizontal sections through MDCK cells injected with these subunits individually, stained with the β_{common} antibody. Right panel: vertical (X–Z sections) through the centre of the cell. These results are representative of the numbers of experiments given in Table 1, with five to eight cells examined per experiment. Scale bar, 10 μm.

Distribution of the β1b/β3 reciprocal chimeras in COS-7 cells

In order to begin to examine the locus for association of β1b to the plasma membrane, we made two chimeras, swapping the C-terminal tail of β1b for β3 between amino acids Q393 and L394 in β1b, which corresponds to positions 341/2 in β3 (Fig. 4). This position was chosen because the C terminus is widely divergent between the two β-subunits, but the locus of the switch is in a sequence LARTLQ-LV in β1b, that is highly conserved in all β-subunits, suggesting that the production of such chimeras will not disrupt the folding of the resultant chimeric proteins.

The chimera β3/1b (with the C terminus of β1b on β3) was clearly membrane-associated in COS-7 cells, similar to the β1b parent (Fig. 4A), whereas the reciprocal chimera β1b/3 (with the C terminus of β3 on β1b) showed a cytoplasmic localization, similar to β3 (Fig. 4B). This clearly implicated the C-terminal region in the plasma membrane localization of β1b. In additional experiments in which we have examined the immuno-localization of co-expressed α1-subunits, we have shown that both chimeras β1b/3 and β3/1b are able to traffic α1A to the plasma membrane in COS-7 cells in the same way as the parental β-subunits (results not shown).

TABLE 1. Distribution of immunostaining for β-subunits in polarized MDCK cells

Subunits injected†	Immunolocalization of β-subunit			
	n	Lateral (%)	Apical (%)	Intracellular (%)
β1b	7	65.5 ± 6.7**	10.3 ± 3.2	24.2 ± 4.6
β2a	3	62.0 ± 3.9**	6.9 ± 0.7	31.1 ± 4.0
β3	5	25.6 ± 3.9	25.6 ± 8.1	48.8 ± 10.2
β4	4	33.9 ± 2.1**	9.1 ± 2.6	63.3 ± 2.9
β1b + α1A/α2-δ	7	17.9 ± 2.7	63.2 ± 3.2**	19.0 ± 2.1
β1b + α1C/α2-δ	5	58.8 ± 7.2*	16.5 ± 5.9	24.7 ± 6.7

†Immunolocalized subunit underlined. For experiments studying the immunolocalization of the β-subunits, injected either alone or with the other subunits, cells were fixed 6 h after microinjection. Quantification was performed on X–Z sections, by determining the per cent of total immunostaining associated with each of the three compartments making up the cell (apical, basolateral and intracellular) using Imagequant software (Molecular Dynamics, Brice & Dolphin, 1999). Results are given as mean ± SEM for the number, *n*, of X–Z sections that were subjected to quantitative analysis. The subunit in each combination, whose immunolocalization was studied is given in bold in the first column of each row. The statistical significances of the differences between apical and basolateral distribution were determined by paired *t*-test: $*P < 0.05$, $**P < 0.01$, given in the column where the greater immunolocalization is observed.

Distribution in COS-7 cells of β1b constructs with deletions and a truncation in the C terminus

As a consequence of the result obtained with the chimeric β1b/β3-subunits, we subsequently made a series of truncations of the β1b C terminus in order to locate the region involved in its plasma membrane association (Fig. 5). A C-terminally truncated β1b construct, terminating at amino acid 569 (β1bΔC570–596) showed similar membrane localization to full-length β1b (Fig. 5A and B), whereas a truncation terminating at amino acid 546 (β1bΔC547–596) showed greater cytoplasmic localization (Fig. 5C), as did a truncation to amino acid 498 (results not shown). A β1b construct with only amino acids 547–556 deleted (β1bΔ547–556) showed similar localization to the β1bΔC547–596 (Fig. 5D). This suggests that the amino acid stretch 547–556 of β1b which contains eight acidic amino acid residues surrounding a single tyrosine (WEEEEDYEEE) is important for the membrane association of β1b.

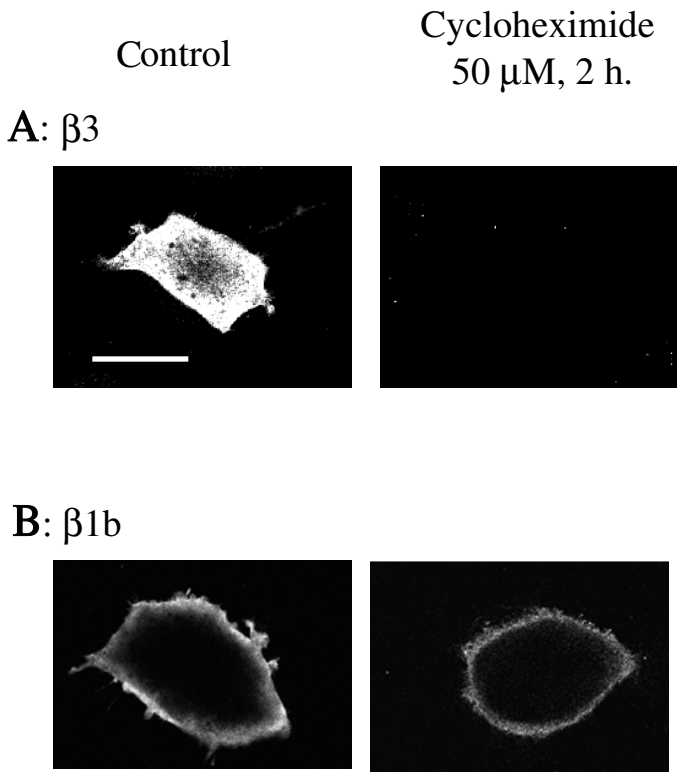


FIG. 3. Effect of cycloheximide on the expression pattern of β1b and β3 in COS-7 cells. (A) Immunolocalization of β3 in cells transfected with only this subunit. (B) Immunolocalization of β1b in cells transfected with only this subunit. Left panel: control experiment performed in parallel but not treated with cycloheximide. Right panel: cells incubated with 50 μM cycloheximide for 2 h at 37 °C, as described in Materials and methods. These results are representative of four different transfections for all conditions, with five to eight cells examined per experiment. Calibration bar, 10 μm.

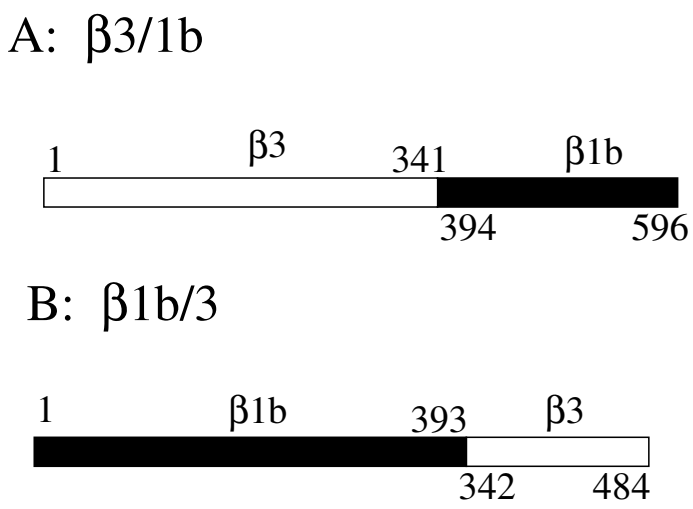
Functional properties of β1b/β3 chimeras and β1b deletion mutants

Electrophysiological experiments show that both the β1b/3 and β3/1b chimeras and the three truncation and deletion constructs are able to support the expression of functional α1B currents in *Xenopus* oocytes (Figs 6 and 7). The currents resulting from co-expression of the parental β1b or β3 are several-fold larger than those resulting from expression of α1B/α2-δ in the absence of an additional β-subunit (Figs 6A–C and 7A). Similar increases in current density were seen for the β1b/3 (Fig. 6D) and β3/1b (Fig. 6E) chimeras, as well as for the β1bΔC547–596, β1bΔC570–596 and β1bΔ547–556 constructs (Fig. 6F–H).

There is also an ~15 mV hyperpolarizing shift in the voltage for 50% activation ($V_{50,act}$, Fig. 7B), and an ~30 mV hyperpolarizing shift in the voltage for 50% steady-state inactivation ($V_{50,inact}$; Fig. 7C–D) for α1Bα2δ currents with any of the β-subunits and constructs, compared with α1B/α2δ in the absence of over-expressed β-subunit. Furthermore, the $V_{50,act}$ and $V_{50,inact}$ values for these constructs were not different from those for the parental β1b- or β3-subunits.

In additional experiments, we have found that inactivation of α1B/α2δ/β1b currents, measured over 4.5 s, can be fitted with a double exponential, giving two time constants of inactivation ($\tau_{inact,1}$ 521 ± 68 ms, and $\tau_{inact,2}$ 2426 ± 357 ms, $n = 3$). The inactivation kinetics of currents resulting from α1B/α2δ co-expressed with β1bΔC547–596, β1bΔC570–596 or β1bΔ547–556, were not different from the parental α1B/α2δ/β1b currents, again suggesting that these constructs all produce similar effects on α1B currents.

Thus, it is clear that while we have identified a motif in these constructs that promotes the membrane association of β1b when it is



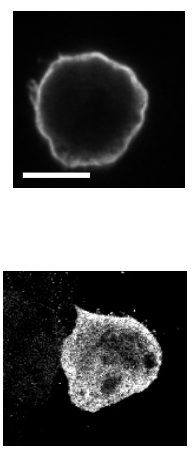


FIG. 4. Distribution of β1b/β3 chimeras in COS-7 cells. (A) Left panel: diagrammatic representation of the β3/1b chimera. Right panel: immunolocalization of this chimera in COS-7 cells transfected only with this construct. These results were obtained using the β1b monoclonal antibody. (B) Left panel: diagrammatic representation of the β1b/3 chimera. Right panel: immunolocalization of this chimera in COS-7 cells transfected only with this construct. These results were obtained using the β3 monoclonal antibody. These results are representative of five and seven different transfections, respectively, with three to five cells examined per experiment. Calibration bar, 10 μm.

expressed alone, nevertheless the presence or absence of this motif does not affect the ability of these constructs to have the expected biophysical effects on calcium channel currents.

Discussion

The VDCC β-subunits are of essential importance to the functioning of VDCCs. They not only traffic α1-subunits to the plasma membrane, but they also affect several aspects of their biophysical properties which are both α1-subunit and β-subunit specific (Birnbaumer *et al*., 1998). VDCC β-subunits have a major binding site on the loop between domains I and II of all α1-subunits (Pragnell *et al*., 1994), and it has also been proposed that there is a C-terminal binding site (Qin *et al*., 1996), although its functional importance has been called into question (Jones *et al*., 1998). A low-affinity N-terminal binding site between α1A and β4 has also been identified (Walker *et al*., 1999), and we have evidence that mutations in the N-terminus of α1B affect VDCC β-subunit-dependent inactivation properties of α1B (Canti *et al*., 1999; Stephens, Page and Dolphin, unpublished results).

There are four β-subunit genes which have conserved and non-conserved regions (Birnbaumer *et al*., 1998). We have shown previously that both β1b and β2a are associated with the plasma membrane when expressed alone in COS-7 cells in the absence of α1-subunits, although they contain no putative plasma membrane spanning α helices (Brice *et al*., 1997). The β2a-subunit is palmitoylated at its N-terminus, and it was proposed that this was responsible for its membrane association (Chien *et al*., 1995). Whereas mutation of the N-terminal cysteine residues which prevented palmitoylation of β2a reduced its effect to slow inactivation of calcium channel currents, it did not prevent its ability to traffic α1-subunits to the plasma membrane (Qin *et al*., 1998). It was initially reported that a palmitoylation-deficient mutant was still membrane-associated (Chien *et al*., 1996), but it was subsequently shown that this was an intracellular membrane rather than the plasmalemma (Chien *et al*., 1998). However, it is clear that palmitoylation is not the only signal required for plasma membrane targeting of β2a, as creation of a chimeric β-subunit with the amino acids 1–16 of rat β2a on β3, while it did become palmitoylated, was not associated with the plasma membrane (Chien *et al*., 1998).

The sequences of all β-subunits include two highly conserved regions that are flanked by a short N-terminal region and a variable length C-terminus and joined by a linker sequence, all of which are divergent between different β-subunits. We have recently shown in a structural modelling study of β1b that it contains a putative N-terminal PDZ domain, associated with an SH3 domain in the first conserved region and a GK domain in the second conserved region, all of which have been involved in protein–protein interactions in a

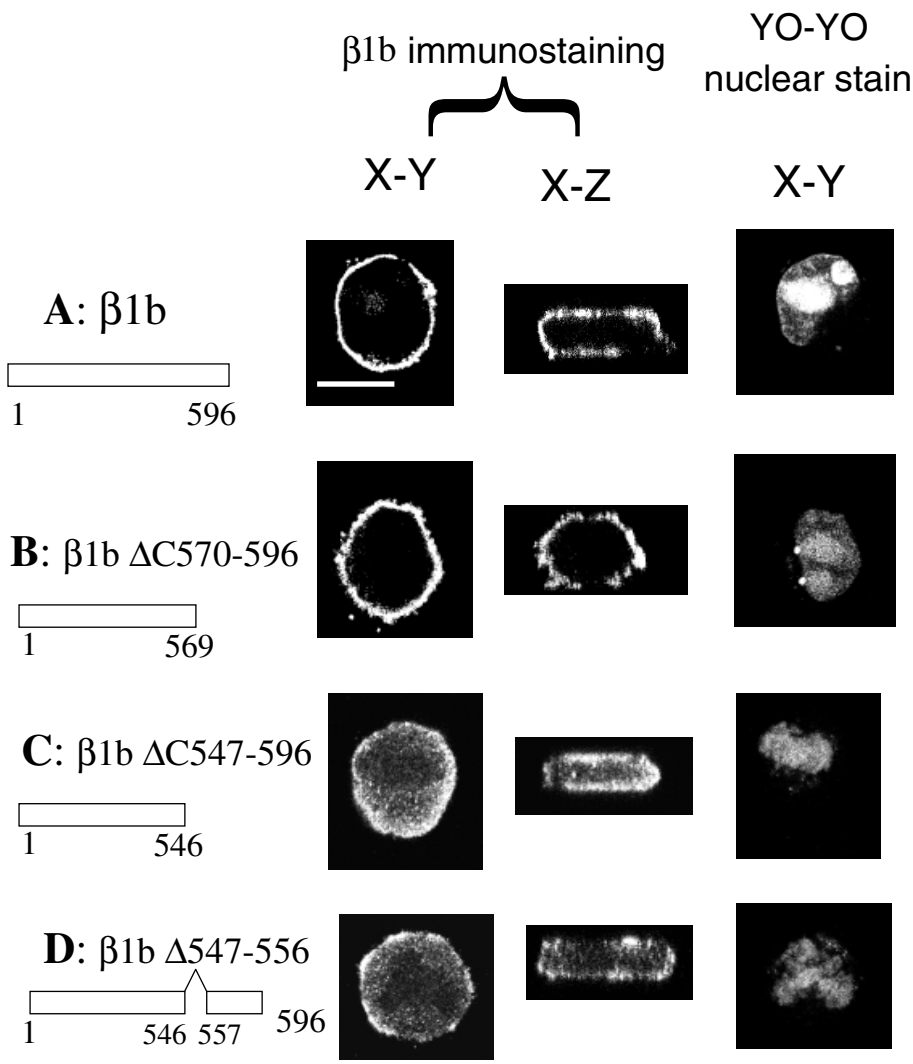


FIG. 5. Distribution of β1b truncations and deletions in COS-7 cells. Immunolocalization for the constructs (A) β1b, (B) β1bΔC570–596, (C) β1bΔC547–596; (D) β1bΔ547–556 in COS-7 cells transfected with these constructs. Left panel: diagrammatic representation of constructs. Middle two panels: immunolocalization for the β-subunit in COS-7 cells. Middle left panel: X–Y section. Middle right panel: X–Z section. Right panel: YO-YO nuclear stain, X–Y section. These results are representative of 15, 15, 14 and 17 different transfections, respectively, with three to five cells examined per experiment. Calibration bar, 10 μm.

wide variety of systems (Hanlon *et al.*, 1999). PDZ domains bind particular C-terminal motifs, SH3 domains bind proline-rich sequences, and GK domains bind, e.g. GKAP (Sheng & Wyszynski, 1997). The family of MAGUK proteins, including PSD-95, are implicated as sub-membrane scaffold proteins in several systems, and are characterized by the presence of a GK domain situated C-terminal to a variable number of PDZ and SH3 domains (Sheng & Wyszynski, 1997; Kim *et al.*, 1998). These three domains, identified in β1b, also appear to be present in the other β-subunits (Hanlon *et al.*, 1999). It is thus possible that one or several of them are involved in targeting calcium channels to specific regions of cells and in their association with other proteins. It might also be hypothesized that one or more of these domains are involved in mediating the plasma membrane association of β1b.

We have observed that β1b is targeted to the plasma membrane when expressed alone, both in COS-7 cells and in the polarized MDCK cell line. In MDCK cells, β1b and β2a were both targeted to the basolateral membrane, when expressed alone, suggesting either that they possess a basolateral sorting signal, or that they bind to a protein that is targeted basolaterally. The basolateral sorting route is thought to require a specific signal, which if absent or deleted in transmembrane proteins results in apical sorting by a default route (Hopkins, 1991). We have shown previously in this cell line that, when α1-subunits are co-expressed with α2-δ and any β, whereas α1B is trafficked to the apical membrane and α1C to the basolateral membrane with all β-subunits, α1A shows differential trafficking with different β-subunits (Brice & Dolphin, 1999). Co-expression with β1b and β4 resulted in α1A transport to the apical membrane, and β2a caused trafficking to the basolateral membrane, with β3 not able to traffic α1A to any plasma membrane (Brice & Dolphin, 1999). The poor ability of β3 to traffic α1A in these cells agrees with the much lower affinity of β3 for the α1A AID sequence, where the binding affinities were measured to be in the rank order β4 > β2a > β1b >> β3 (De Waard *et al.*, 1995).

In the present study we have shown that in MDCK cells the sorting of β1b is influenced by the α1-subunit with which it is expressed. When co-expressed with α1A and α2-δ, β1b was found primarily at the apical membrane of MDCK cells, the same location as both α1A (Brice & Dolphin, 1999) and α2-δ (Brice and Dolphin, unpublished results) when expressed in this combination. In contrast, when co-expressed with α1C and α2-δ, β1b was found primarily at the basolateral membrane, like α1C when co-expressed in this combination (Brice & Dolphin, 1999), as was β1b when expressed alone.

We have also observed that the β3 protein, which is associated with an intracellular membrane-bound compartment when expressed alone (Campbell *et al.*, 1995), was rapidly turned over, because immunostaining was completely lost after 2–6 h treatment with the protein synthesis inhibitor cycloheximide. It is possible that β3 expressed alone is retained in association with intracellular membranes, possibly mis-folded and bound to chaperone proteins, and as such is a target for proteolytic

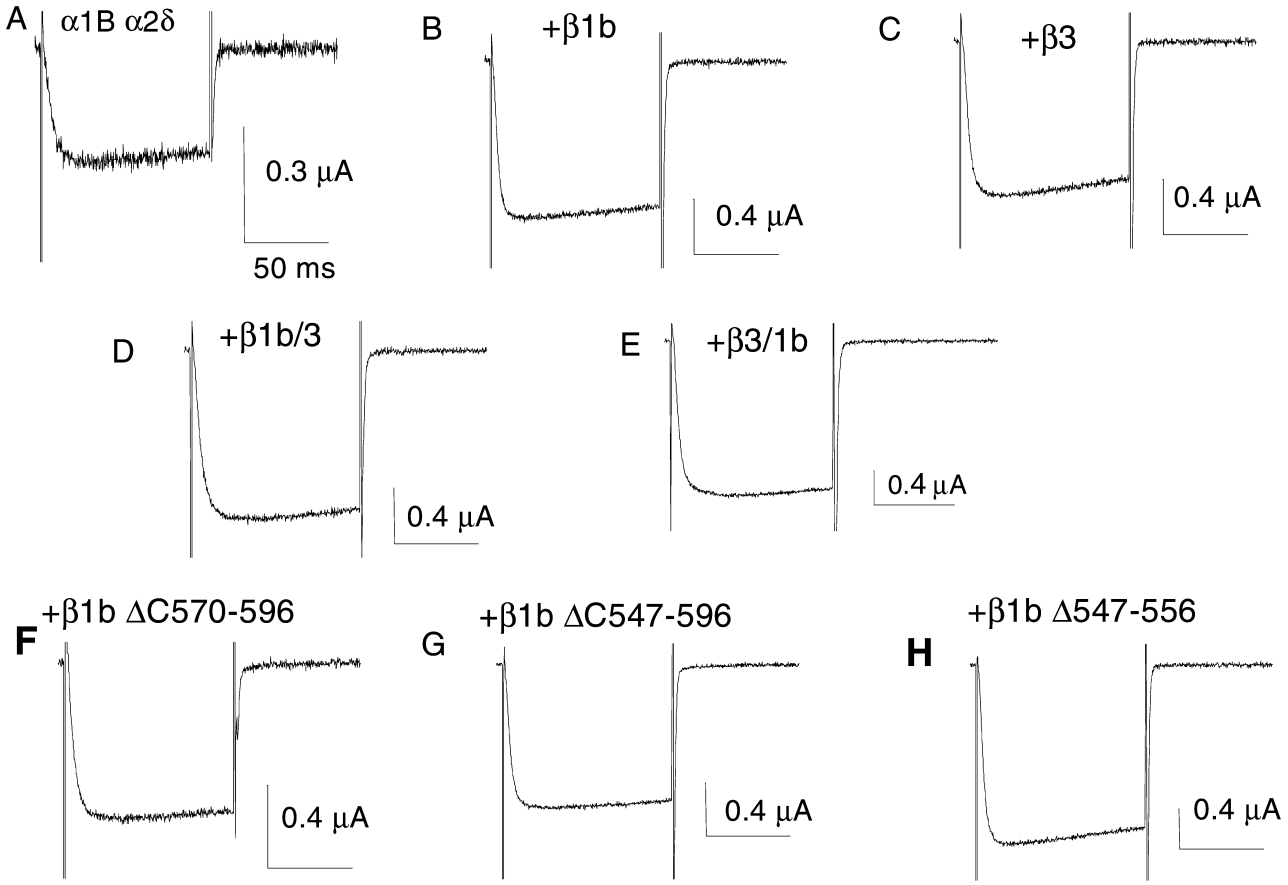


FIG. 6. The β1b/β3 chimeras and the β1b truncation and deletion constructs can support functional α1B currents. Representative current traces are shown for the channel combinations given above each trace (A) α1B/α2δ alone, (B) +β1b, (C) +β3, (D) +β1b/3, (E) +β3/1b, (F) +β1bΔC570–596, (G) +β1bΔC547–596, (H) +β1bΔ547–556. The calibration bars are for the current amplitude shown beside each trace and for 50 ms.

enzymes (Hartl, 1996). In contrast, plasma membrane-associated β1b is much more stable, as its immunolocalization is not affected by inhibition of protein synthesis for up to 6 h. Thus, it appears that association with a target at the plasma membrane has protected the β1b-subunit from proteolytic breakdown.

We subsequently made reciprocal chimeras between β1b, which associates with the plasma membrane, and β3, which does not. The chimeras were made by switching at a point in the C-terminus beyond the second conserved region. The two chimeras produced the clear result that the sequence involved in the membrane association of β1b was in its C-terminal region, which shows low homology between the different β-subunits, thus ruling out a role for the PDZ, SH3 and GK domains, because these are all situated N-terminal to the site at which the chimeric switch was made.

Subsequent production of β1b constructs, truncated at various positions in the C-terminus, and a deletion mutation of β1b containing a largely acidic motif (amino acids 547–556, WEEEEDYEEE), identifies this amino acid motif as being involved in the membrane-association of β1b. Acidic motifs in other proteins have been found to bind to pleckstrin homology (PH) domains (Burks *et al*., 1998). However, the binding partner target of this β1b motif remains to be determined. In the non-membrane-associated β3- and β4-subunits, the homologous amino acids are RHL**EEDY**A*D*A and PLV**EEDY**P*D*S, with identical residues in bold and conserved charge in italics. This residual homology is apparently insufficient to support association with the plasma membrane. No homologous sequence is present in the C-terminal region of β2a, where palmitoylation plays a role in its membrane association.

In conclusion, we have identified a motif in β1b that is essential for its plasma membrane association, but is not essential for the basic properties of this β-subunit to traffic α1-subunits to the plasma membrane, or to support functional α1/β calcium channel currents. It is therefore possible that this motif is involved in native tissues in the association of β1b-containing calcium channels with other elements concerned with their clustering or localization. Thus, if certain β-subunits are more mobile than others, this would begin to provide a rationale for the existence of three β-subunits (β1b, β3 and β4) which otherwise all have very similar effects on calcium channel biophysical properties.

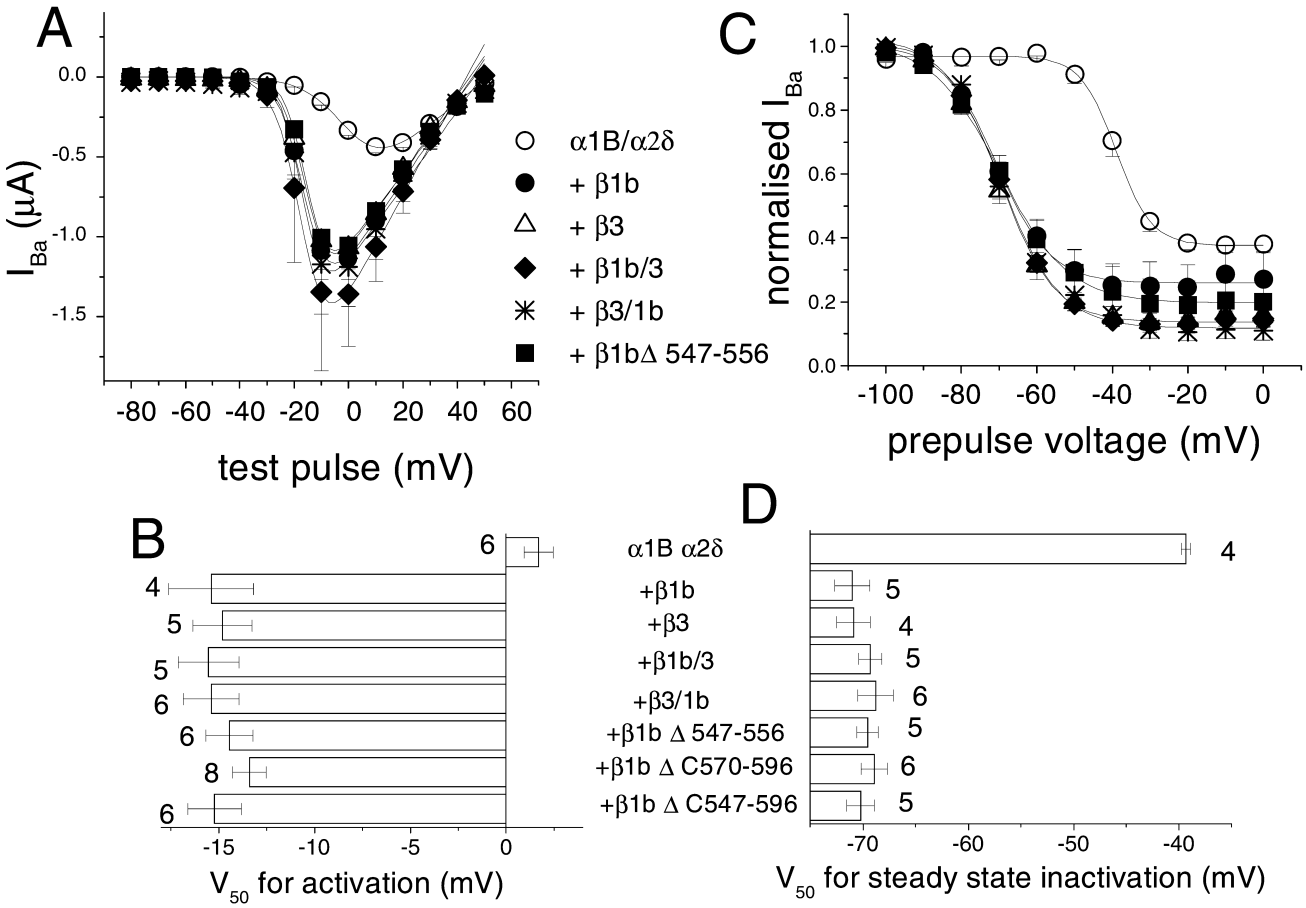


FIG. 7. The activation and steady-state inactivation properties of the currents produced by α1B/α2δ co-expressed with β1b/β3 chimeras and the β1b truncation and deletion constructs. (A) Current–voltage relationships (mean ± SEM) for the combination of α1B/α2δ with no expressed β-subunit or with the parental β1b- or β3-subunits or the constructs as shown in Fig. 6. For clarity the data for the two truncation constructs β1bΔC570–596 and β1bΔC547–596 are not plotted as they showed essentially the same current–voltage characteristics as β1bΔ547–556. The lines are the fits obtained by fitting a modified Boltzmann equation to the mean current–voltage relationships (Canti *et al*., 1999). (B) Histogram of $V_{50,act}$ (mean ± SEM) for the data shown in (A), obtained by fitting a modified Boltzmann equation to the individual current–voltage relationship data, for the number of experiments given beside each bar. (C) Normalized steady-state inactivation curves (mean ± SEM) for the combination of α1B/α2δ with the parental β1b- or β3-subunits or the constructs as shown in Fig. 6. For clarity the data for the two truncation constructs β1bΔC570–596 and β1bΔC547–596 are not shown as they showed essentially the same inactivation characteristics as β1bΔ547–556. The lines are the fits obtained by fitting a Boltzmann equation to the mean data. (D) Histogram of $V_{50,inact}$ (mean ± SEM) for the data shown in (C), obtained by fitting a Boltzmann equation to the individual steady-state inactivation curves, for the number of experiments given beside each bar.

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Abbreviations

AID, α-interaction domain; BID, β-interaction domain; BSA, bovine serum albumin; DMEM, Dulbecco's minimum essential medium; GK, guanylate kinase; MAGUK, membrane-associated guanylate kinase proteins; MDCK, Madin Darby canine kidney cells; SH, *src* homology; TBS, Tris-buffered saline; VDCCs, voltage-dependent calcium currents.

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