Antisense depletion of β-subunits modulates the biophysical and pharmacological properties of neuronal calcium channels

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1. The role of the voltage-dependent calcium channel (VDCC) β-subunit has been examined in cultured rat dorsal root ganglion neurones (DRGs). An antipeptide antibody was raised and this recognized proteins corresponding to β-subunits in a number of preparations. Immunoreactivity for the VDCC β-subunit in DRGs was concentrated on the internal side of the plasma membrane but was also present in the cytoplasm.

2. A twenty-six-mer antisense oligonucleotide with homology to all published VDCC β-subunit sequences was microinjected into individual cells, and maximal depletion of VDCC β-subunit immunoreactivity was observed after 108 h suggesting a half-life for the turnover of the β-subunit greater than 50 h. No depletion was obtained with nonsense oligonucleotide.

3. The effect of depletion of VDCC β-subunit immunoreactivity on calcium channel currents in these cells was a reduction in amplitude of the maximum current of about 47%, and a shift in the voltage dependence of current activation of about +7 mV. These effects are the converse of those observed following co-expression of cloned β- with α_1-subunits in oocytes and other expression systems.

4. The ability of the 1,4-dihydropyridine (DHP) agonist Bay K 8644 to enhance calcium channel currents was greatly reduced following depletion of β-subunit immunoreactivity. This result is in agreement with the finding in several systems that co-expression of the β-subunit with α_1-subunits results in an increased number of DHP binding sites.

5. These results show that calcium channel β-subunits form part of native neuronal calcium channels and modify their biophysical and pharmacological properties.

Voltage-dependent calcium channels (VDCCs) when purified from skeletal muscle consist of five subunits, the α_1-subunit which binds 1,4-dihydropyridines (DHPs) and forms the Ca^{2+} channel pore and four ancillary subunits α_2, δ, β and γ (Takahashi, Seager, Jones, Reber & Catterall, 1987; Catterall, Seagar & Takahashi, 1988; Ruth *et al.* 1989; De Jongh, Warner & Catterall, 1990; Snutch & Reiner, 1992). Cloning of the genes for these proteins has revealed much diversity, not only in the α_1-subunits, but also in the co-purifying subunits (for review see Snutch & Reiner, 1992). A number of other α_1-subunit genes have been cloned, some of which code for subtypes of DHP-sensitive L-type channels in other tissues (Ahlijanian, Westenbroek & Catterall, 1990; Hui, Ellinor, Krizanova, Wang, Diebold & Schwartz, 1991) while others are thought to code for distinct classes of VDCC (Snutch, Leonard, Gilbert, Lester & Davidson, 1990; Dubel *et al.* 1992; Williams *et al.* 1992). The N-type VDCC, first identified electrophysiologically (Fox, Nowycky & Tsien, 1987), is characterized by irreversible blockade by ω-conotoxin GVIA (ω-CTX) (Plummer, Logothetis & Hess, 1989). The B clone identified by Snutch *et al.* (1990) codes for a protein that binds ω-CTX (Dubel *et al.* 1992) and when expressed produces a calcium current that is blocked by this toxin. There is evidence that ω-CTX receptor is also associated with a β-subunit (McEnery, Snowman & Snyder, 1991; Sakamoto & Campbell, 1991; Witcher, De Waard & Campbell, 1993). Thus, it is likely that these additional subunits form part of other VDCCs as well as the L-type. There is evidence from co-expression studies in oocytes and mammalian cells that the α_2- and β-subunits modify the biophysical and pharmacological characteristics of the current observed on expression of the α_1-subunits alone (Singer, Biel, Lotan, Flockerzi, Hofmann & Dascal, 1991;

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Itagaki, Koch, Bodi, Klöckner, Slish & Schwartz, 1992; Williams *et al.* 1992; Stea, Dubel, Pragnell, Leonard, Campbell & Snutch, 1993; Zhang *et al.* 1993). A spectrum of effects has been attributed to the β-subunit, including an increase in the current amplitude (Singer *et al.* 1991; Hullin *et al.* 1992; Lory, Varadi, Slish, Varadi & Schwartz, 1993; Stea *et al.* 1993), increase in the kinetics of activation and inactivation of the current (Varadi, Lory, Schultz, Varadi & Schwartz, 1991) and a hyperpolarizing shift in voltage dependence of activation of the current (Singer *et al.* 1991; Perez-Reyes *et al.* 1992; Castellano, Wei, Birnbaumer & Perez-Reyes, 1993*a*, *b*; Stea *et al.* 1993). However, these effects have not been observed in all studies, and may depend on the degree of expression of the β-subunit (Varadi *et al.* 1991), and presence of other subunits (Singer *et al.* 1991).

We have now examined the effect of depletion of the β-subunit on VDCCs in intact primary cultured rat dorsal root ganglion neurones (DRGs) which contain N-, L- and P-subtypes of high threshold VDCC (Mintz, Adams & Bean, 1992; A. C. Dolphin, unpublished results). We have used the technique of microinjection of an antisense oligonucleotide complementary to the sequences of the four known β-subunits, a strategy that has been used previously to deplete a number of different proteins in cultured cells (Kleuss, Hescheler, Ewel, Rosenthal, Schultz & Wittig, 1991; Campbell, Berrow & Dolphin, 1993; Lledo, Vernier, Vincent, Mason & Zorec, 1993).

METHODS

Tissue culture

Dorsal root ganglia were dissected from 2-day-old rats killed by decapitation, incubated with collagenase (1·25 μg ml^{-1}) for 13 min and then for 6 min with trypsin (2·5 μg ml^{-1}) in Ham's F14 medium (Imperial, Andover, UK) containing 10% heat-inactivated horse serum, glutamine (2 mM), penicillin (50 i.u. ml^{-1}) and streptomycin (50 μg ml^{-1}). The ganglia were then mechanically dissociated with a fire-polished pipette in the presence of DNase (1600 Kunitz ml^{-1}). DRG neurones were plated on polyornithine-laminin at a density of 1–3 × 10^4 cells per 2·2 cm^2 square coverslip in the above medium containing nerve growth factor (10 ng ml^{-1}). After 2 days in culture at 37 °C in air containing 5% CO_2, the cells were incubated with cytosine-arabino-furanoside (7 ng ml^{-1}) for 48 h. The medium was thereafter changed every 3–4 days. Cells were used after 10–15 days in culture.

Anti-VDCC β-subunit antisera

The β-subunit antiserum (β 2491) used in this study was raised against a penta-decapeptide corresponding to amino acids 65–79 inclusive of the deduced sequence of the rat brain β_{1b}-subunit (Pragnell, Sakamoto, Jay & Campbell, 1991). This peptide sequence (SRPSDSDVSLEEDRE) is common to all current rat brain β-subunit clones with the exception of β_3 which has a single leucine insertion (Castellano *et al.* 1993*b*). Polyclonal antiserum to this peptide (β 2491) was generated in rabbits using standard techniques, and cross-reacted in ELISA tests with both the immunizing peptide and the corresponding β_3-peptide (SRPSLDSDVSLEEDRE).

Other antisera

The Gα_o antiserum was raised as described in Campbell *et al.* (1993). The VDCC α_2-antiserum was raised against a peptide (SLEDIKRLTPRFTLC). It recognized a single band of approximate mol. mass of 140 kDa on immunoblots of purified rat skeletal muscle T-tubules, rabbit DHP receptor, and brain membranes. It will be characterized in detail elsewhere (K. Brickley, V. Campell, N. Berrow, R. Leech, R. I. Dorman, D. Wray, A. C. Dolphin & S. A. Baldwin, unpublished results).

Immunoblotting

Rat T-tubules were prepared from rat thigh muscle and purified DHP receptor was prepared from rabbit thigh muscle as previously described (Rosemblatt, Hidalgo, Vergara & Ikemoto, 1981; Flockerzi, Oeken & Hofmann, 1986). Samples were diluted to approximately 2 mg protein ml^{-1} with sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris, pH 6·8, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 4% w/v SDS and 0·002% w/v Bromophenol Blue) and heated to 100 °C for 5 min. T-tubule proteins (50 μg per lane) were separated by electrophoresis on 4–12% polyacrylamide minigels, transferred to polyvinylidene difluoride (PVDF) membranes (ProBlott, Applied Biosystems Inc., UK) and immunoblotted with either pre-immune or anti-β-subunit antiserum. Immunoreactive bands were visualized using anti-rabbit IgG–horseradish peroxidase conjugate and amino ethyl carbazole (Sigma, UK). Standards were used to determine the molecular weights of individual protein bands in this system.

Affinity purification of β 2491 antibody

Peptide-specific antibodies were purified from crude serum by chromatography against the immunizing peptide (2 mg) immobilized on a Sepharose matrix (Sulfolink, Pierce, Chester, UK). All steps were carried out at 4 °C. Serum samples were diluted 2-fold in 10 mM Na_2HPO_4, 145 mM NaCl, pH 7·2 (phosphate-buffered saline, PBS) and repeatedly passed through the peptide column for 2 h. The column was then washed with 10 mM Na_2HPO_4, 800 mM NaCl at pH 7·2 to remove non-specifically bound protein. Bound IgG was then eluted in 5 M $MgCl_2$, followed immediately by 10-fold dilution in distilled water. Following dialysis *versus* PBS, purified antibodies were concentrated using a Centricon 30 concentrator (Amicon, Stonehouse, UK) and stored at −70 °C.

Production of β 2491 antibody affinity matrix

Purified antibody (240 μg) was bound to protein A–Sepharose (10 mg) in 0·1 M borate at pH 8·2 for 3 h at 20 °C with agitation. After washing in 0·2 M triethanolamine, pH 8·2, the bound IgG was covalently coupled to the gel by resuspension in 20 mM dimethylpimelimidate in 0·2 M triethanolamine, pH 8·2 for 16 h at 4 °C. The gel was then washed in the following buffers: 20 mM ethanolamine at pH 8·2, 0·1 M borate at pH 8·2 and 5 M $MgCl_2$ to remove non-covalently bound IgG. The gel was then diluted with Sepharose CL-4B (0·5 ml) and washed in PBS.

Purification of β 2491 antibody-immunoreactive protein from brain membranes

Brains were dissected from six rats (Sprague–Dawley males > 250 g) and visible white matter was removed at 4 °C in homogenizing buffer (10 mM Hepes, pH 7·4, containing

5 mM benzamidine, 5 mM dithiothreitol, 0·1% (w/v) soy bean trypsin inhibitor, 100 μM phenyl-methyl-sulphonyl fluoride (MSF), 50 μM leupeptin and 25 trypsin inhibitor units ml^{-1} aprotinin). Brains were roughly chopped in buffer prior to homogenization in a Teflon and/or glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min, the pellet discarded and the supernatant re-centrifuged at 4000 *g* for 10 min; membranes were then isolated from the supernatant by centrifugation at 50 000 *g* for 30 min.

The membranes were resuspended with sonication, in 1% Nonidet P-40 (BDH, Leicester, UK) in homogenizing buffer at a protein concentration of 2 mg ml^{-1} and incubated for 90 min at 4 °C with agitation. Insoluble material was collected at 100 000 *g* for 30 min and the soluble supernatant was then incubated with the β2491 antibody gel for 16 h at 4 °C with agitation. The gel was packed in a column and washed in homogenizing buffer followed by 10 mM Na_2HPO_4, 800 mM NaCl at pH 7·2 to remove non-specifically bound material. Antibody-bound material was eluted in 5 M $MgCl_2$ and the column washed in distilled water. The eluate was dialysed in 3500 mol. wt cut-off membrane (Spectra-Por, Pierce) against excess ultra-high quality water and concentrated by freeze drying.

Proteins eluted from the β2491 antibody column were then separated and transferred to PVDF membranes (as described under Immunoblotting, with the exception that a 10% polyacrylamide minigel was used). Immobilized proteins were stained using 0·5% Coomassie Blue G in 7% acetic acid; destaining was achieved by rinsing the membranes thoroughly in methanol.

Immunocytochemistry

Skeletal muscle. Frozen sections (10 μm) of rat skeletal muscle were cut and attached to glass slides using standard techniques. Sections were washed with 154 mM NaCl buffered with 20 mM sodium phosphate pH 7·4 (PBS) and incubated with anti-β-subunit antiserum or pre-immune serum for 3·5 h at 20 °C. Sections were then washed in PBS (3 × 5 min) and incubated with a 1:40 dilution of swine anti-rabbit IgG conjugated to rhodamine for 1 h at 20 °C. Sections were then washed (5 × 3 min) and mounted in antifade mountant (Citifluor, City University, London). They were viewed with a confocal laser scanning microscope (Biorad, Hemel Hempstead, UK).

Cultured DRGs. Cells were washed with 154 mM NaCl containing 40 mM Tris pH 7·4 (TBS), fixed with 4% paraformaldehyde in TBS for 30 min at room temperature, and in most experiments were permeabilized with 0·02% Triton X-100 in TBS for 3 × 5 min. Cells were then washed (3 × 5 min) with TBS containing 20% goat serum, 4% bovine serum albumin and 0·1% DL-lysine, and incubated with a 1:2500 dilution in the same solution of anti-β-subunit antiserum, overnight at 4 °C. The cells were washed (4 × 5 min) and incubated with goat anti-rabbit IgG conjugated to biotin (1:200) for 2 h at 4 °C. Cells were washed (4 × 5 min) and incubated for a further 2 h at 4 °C with extravidin conjugated to fluorescein (1:50; both reagents from Sigma). The cells were washed (5 × 5 min) and mounted in antifade mountant and viewed with a confocal miscroscope. Images were taken under conditions of constant illumination of the laser, without prior laser exposure. To determine the degree of immunofluorescence associated with the cell, all images were enhanced equally to make best use of the total range of grey values (0–255). Using the image analysis facility, the average grey value intensity (GVI) was obtained for each fluorescent image. This process was performed with all data acquisition parameters (aperture, gain, grey value ramp) constant for all cells.

Antisense oligonucleotides

Phosphorothioate deoxyoligonucleotides (Oswel, Edinburgh) were used in these experiments. The β-subunit antisense oligonucleotide had the sequence:

5′ACCAGCCTTCCGATCCACCAGTCATT3′.

This is 96% complementary to nucleotides 487–511 of the β_{1b}-mRNA (Pragnell *et al.* 1991), and 92% complementary to nucleotides 676–701 of the β_2 mRNA (Perez-Reyes *et al.* 1992), 394–419 of the β_3 mRNA (Castellano *et al.* 1993*b*) and 397–422 of the β_4 mRNA (Castellano *et al.* 1993*a*). The only sequences found within the EMBL and/or Genbank databases to have significant homology to the β-subunit antisense target sequences were those encoding other VDCC β-subunits. The nonsense oligonucleotide had the sequence:

5′GAAGTAGGTCTTGGTGGTGG3′.

It has no target sequence of significant homology within the EMBL/Genbank databases. Most experiments were performed using the nonsense sequence as control, but identical results were obtained with the following scrambled antisense sequence:

5′AACGCTTACGTCCACCTACTTACCCG3′.

Microinjection

To identify DRGs following microinjection, cells were grown on coverslips with a 2 mm circle etched in the centre; all cells within the circle were injected. Cells were microinjected using an Eppendorf microinjector with approximately 20 fl of oligonucleotide representing about 5×10^6 copies, using pipettes of tip diameter 0·5 μm, an injection time of 0·1 s and a pressure of 20 kPa. The injection volume was estimated using tritiated tracer molecules. Cells were returned to the incubator after microinjection. The injection target was the cell cytoplasm, since oligonucleotides accumulate rapidly in the nucleus following cytoplasmic microinjection (Leonatti, Mechti, Degols, Gagnor & Lebleu, 1991). The success of intracellular injections was verified in several experiments by the injection of fluorescein isothiocyanate-conjugated dextran.

Electrophysiology

Electrophysiological recordings were made using a patch pipette solution containing (mM): caesium aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0·1; K_2ATP, 2·0; Hepes, 10; pH 7·2, 310 mosmol with sucrose. The external solution contained (mM): TEA-Br, 160; KCl, 3; $NaHCO_3$, 1·0; $MgCl_2$, 1·0; Hepes, 10; glucose, 4; $BaCl_2$, 1; and tetrodotoxin, 0·0005; pH 7·4, 320 mosmol with sucrose. Patch pipettes (2–4 MΩ resistance) were pulled from borosilicate glass, fire-polished and coated with Sigma-cote (Sigma). An Axopatch 1D amplifier was used and recordings were filtered at 1 or 2 kHz (4-pole Bessel filter) and digitized at 5–44 kHz using a Digidata A/D converter. Analysis was performed using pCLAMP 5 or 6 and currents were leak subtracted using a P/5 protocol. The holding current at −80 mV was normally less than 0·05 nA and the series resistance was normally less than 5 MΩ. Step potentials have been corrected for series resistance errors. All electrophysiological

recordings were made without knowledge of the microinjection treatment. Racemic Bay K 8644 was obtained from Bayer (Germany).

RESULTS

Characterization of the β-subunit antipeptide antiserum

An antipeptide antiserum was raised against a peptide sequence common to β_1, β_2 and β_4 and with one amino acid deletion compared with the corresponding β_3 sequence. Pre-immune serum did not recognize any proteins on immunoblots (Fig. 1*A a* and *b*). The antiserum recognized a protein of about 52 kDa in rat skeletal muscle T-tubules (Fig. 1*A c*) and a protein of 55 kDa in DHP receptor purified from rabbit skeletal muscle (Fig. 1*A d*). An immunoaffinity column was prepared using the antibody purified from this antiserum and the protein retained on the column from NP-40-solubilized rat brain membranes was eluted with 5 M Mg^{2+}. The eluted material had a mol. mass of approximately 65 kDa (Fig. 1*B*), the same as the predicted mol. mass of the VDCC β_{1b}-subunit (Pragnell *et al.* 1991). Because the band was very faint it could not be demonstrated by this technique that the antibody recognized all four β-subunits present in brain. However, this is likely because it was raised to a conserved sequence. In agreement with this, the antiserum was also able to immunoprecipitate a protein with a mol. mass of approximately 52 kDa from iodinated NP-40-solubilized rat brain membranes (results not shown). This may correspond to the β_3-subunit.

Confocal examination of frozen sections from rat skeletal muscle showed immunolocalization of antigen to T-tubules with a repeat sequence of 1·8 μm (Fig. 2*A*). A similar immunolocalization was observed for skeletal muscle α_1- and α_2-subunits (K. Brickley, A. C. Dolphin, R. I. Norman & S. A. Baldwin, unpublished results). The confocal immunocytochemical localization of the β-subunit in cultured DRGs showed intracellular distribution, concentrated beneath the plasma membrane (Fig. 2*B*). Neurites were stained but are not shown because the confocal section is taken approximately 10 μm up from the coverslip. There was no staining of non-neuronal cells. No staining was observed unless cells were permeabilized following fixation (results not shown). Staining was reduced by pre-absorption of the antibody with immunizing peptide (Fig. 2*C*) to levels similar to those obtained with pre-immune serum (Fig. 2*D*).

Microinjection of antisense oligonucleotide complementary to β-subunit mRNA

Microinjection of β-subunit antisense oligonucleotide produced a large reduction in calcium channel β-subunit immunoreactivity at 108 h after injection (Fig. 2*E*). No depletion of β-subunit immunoreactivity was observed at

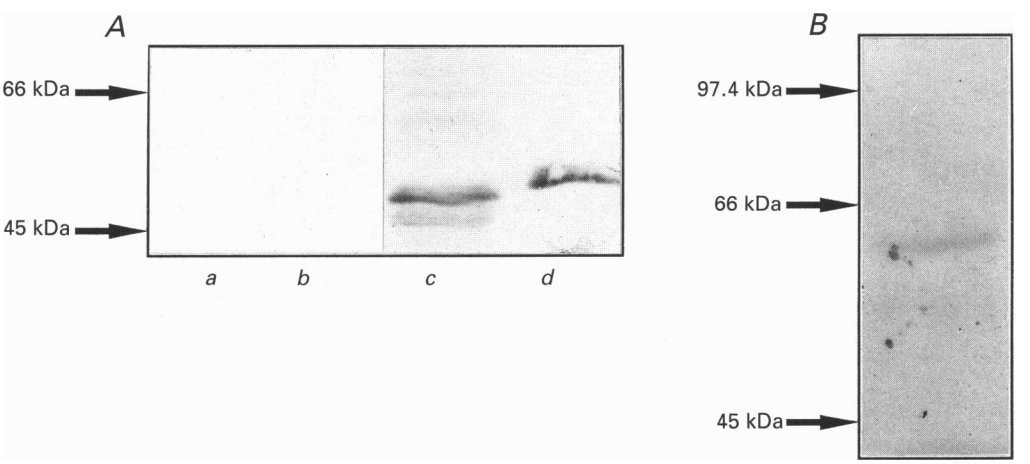

Figure 1. Characterization of the anti-VDCC β-subunit

A, the anti-β-subunit antiserum β2491 (1 : 500 dilution) is immunoreactive with a protein of approximately 52 kDa in rat skeletal muscle T-tubules (50 μg, *c*) and a protein of 55 kDa in purified rabbit skeletal muscle DHP receptor (1 μg, *d*). Pre-immune serum (1:500 dilution) shows no immunoreactivity to either rat skeletal muscle T-tubules (50 μg, *a*) or purified rabbit skeletal muscle DHP receptor (1 μg, *b*). Positions of mol. mass standards in this gel system are indicated. *B*, the β2491 antibody purifies a protein from NP-40-solubilized rat brain membranes with a mol. mass of approximately 65 kDa. Positions of mol. mass standards in this gel system are indicated.

the same time after microinjection of a nonsense sequence (Figs 2*F* and 3*A*). The time course of depletion of β-subunit immunoreactivity was examined by quantifying the total amount of staining in cells at increasing times after β-subunit antisense microinjection. The maximum depletion (about 93%) was between 108 and 130 h after microinjection, after which time immunostaining for β-subunit began to recover (Fig. 3*A*). This suggests a half-life for β-subunit turnover greater than 50 h. In contrast, the time course of depletion of the G protein α_o-subunit following microinjection of an antisense oligonucleotide complementary to both α_{o1} and α_{o2} (Campbell, Berrow & Dolphin, 1993) was much more rapid (Fig. 3*B*) indicating a half-life for turnover of $G\alpha_o$ of about 16 h.

To examine whether the β-antisense oligonucleotide caused a non-specific reduction of the synthesis of other proteins involved in the VDCC complex or in signal transduction involving the $GABA_B$ receptor, we examined immunocytochemically the levels of $G\alpha_o$ and VDCC α_2. Depletion of VDCC β-subunit immunoreactivity had no effect on the

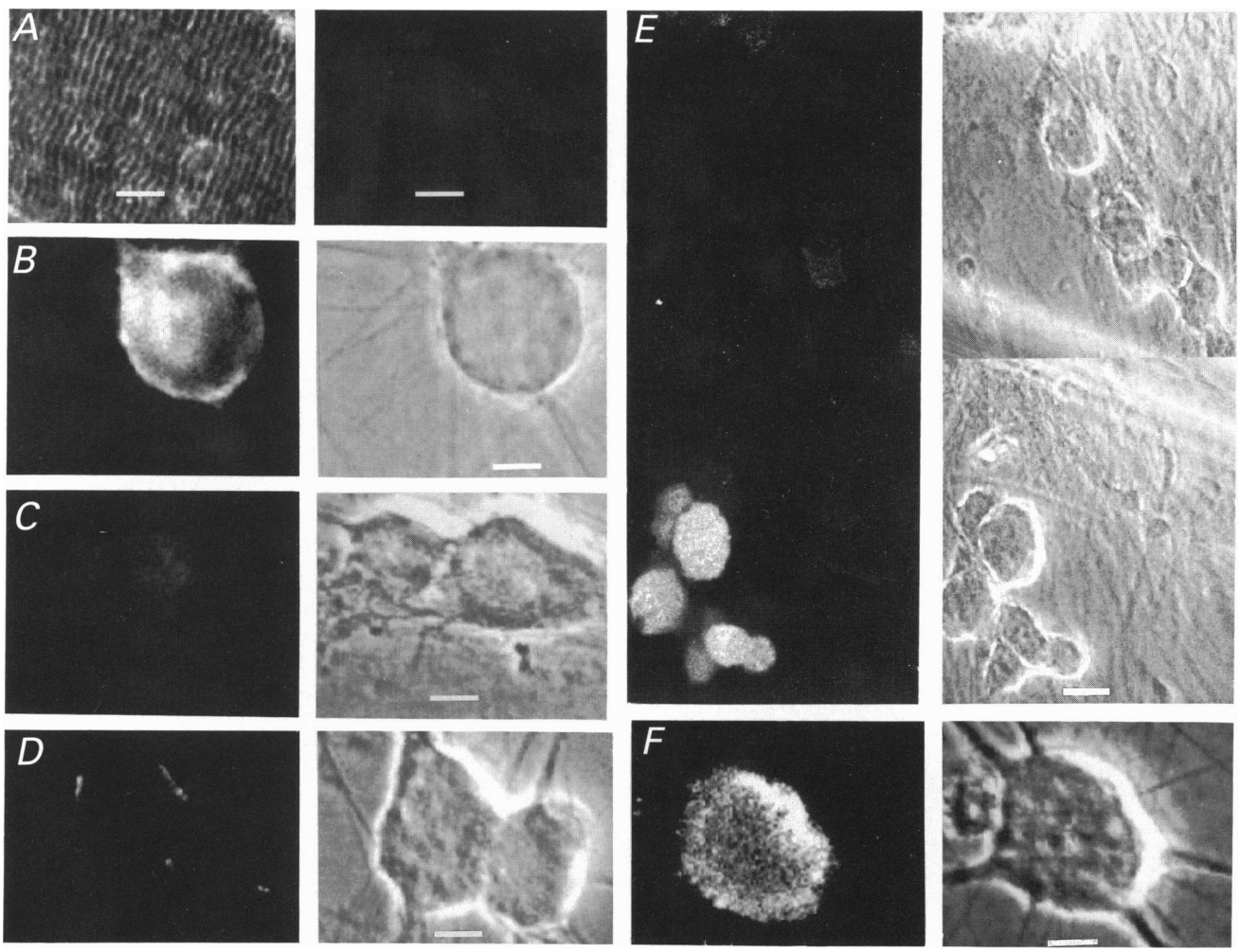

Figure 2. Confocal immunocytochemical localization of VDCC β-subunit immunoreactivity in skeletal muscle and DRGs

A, immunostaining for β-subunit in 10 μm frozen sections of rat skeletal muscle (left). Lack of staining with pre-immune serum (right). *B*, immunostaining for β-subunit in cultured rat DRGs, showing localization to sub-plasma membrane region. *C*, reduction in immunostaining for β-subunit in DRGs, following pre-absorption of antiserum for 1 h with immunizing peptide (100 μM). *D*, lack of staining in DRGs with pre-immune serum. *E*, cells above the etched mark were injected with β-subunit antisense oligonucleotide 108 h previously, whereas cells below the mark were not injected. Immunostaining for β-subunit as in *B*. *F*, lack of effect of injection 108 h previously of nonsense oligonucleotide on β-subunit immunoreactivity. *B–F*, fluorescence image on left is a 1 μm confocal section through the cell about 10 μm up from the attachment plaque; phase image is on right. Scale bars: *A–F*, 5 μm; *E*, 10 μm.

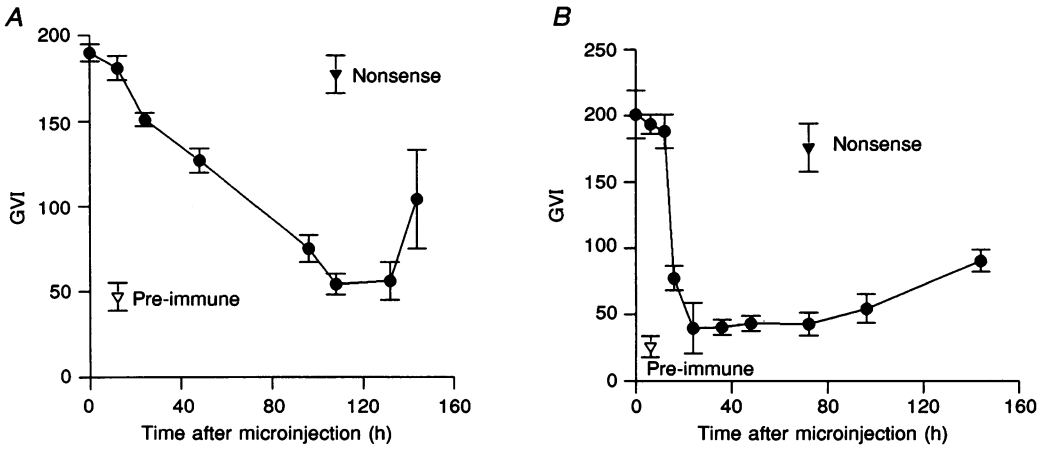

Figure 3. Depletion of VDCC β-subunit and $G\alpha_o$ immunoreactivity

Time course of the depletion of VDCC β-subunit immunoreactivity following β-subunit antisense oligonucleotide injection: comparison with the effect of $G\alpha_o$ antisense oligonucleotide injection. Antisense oligonucleotides complementary to β-subunit mRNA (*A*) or $G\alpha_o$ mRNA (*B*) were microinjected at time 0, and the immunoreactivity quantified at varying times thereafter (●), as described in Methods for the β-subunit and in Campell *et al.* (1993) for $G\alpha_o$. The lack of effect of the nonsense oligonucleotide is shown at the time of peak depletion by the antisense oligonucleotide (▼) and the level of staining shown by the pre-immune serum (▽) is shown at time 0.

level of $G\alpha_o$ (Fig. 4*A*) or VDCC α_2 (Fig. 4*B*) immunoreactivity at either 24 h (not shown) or 108 h after microinjection of the β-subunit antisense oligonucleotide. Similarly depletion of $G\alpha_o$ as in Campbell *et al.* (1993) had no effect on the level of β-subunit detected immunocytochemically at either 24 or 108 h after microinjection of the $G\alpha_o$ antisense oligonucleotide (results not shown).

Effect of depletion of β subunit immunoreactivity on biophysical properties of calcium channel currents

Calcium channel currents (I_{Ba}) were examined in cells injected with the β-antisense oligonucleotide, and compared with control non-injected cells and those injected with the nonsense oligonucleotide, both at 108–116 h following injection. Current–voltage relationships were

Table 1. Effect of depletion of VDCC β-subunit on biophysical properties of I_{Ba} in DRGs

	β-Antisense	Nonsense	Control
I_{max} (80 ms step)			
Capacitance (pF)	23·3 ± 2·4 (16)	24·5 ± 3·8 (12)	24·7 ± 5·1 (5)
Peak amplitude (pA pF^{-1})	−26·6 ± 2·3* (16)	−49·8 ± 6·6 (12)	−47·9 ± 5·4 (5)
Percentage inactivation	21·4 ± 3·3 * (16)	14·3 ± 3·4 (12)	14·8 ± 5·7 (5)
Time to peak (ms)	12·4 ± 1·0 (16)	12·3 ± 1·6 (12)	20·2 ± 9·8 (5)
From *I–V* relationships			
$V_{50\ activation}$ (mV)	+9·9 ± 1·9* (14)	+1·7 ± 2·3 (11)	+2·01 ± 2·00 (11)
k (slope factor)	5·26 ± 0·42 (14)	4·87 ± 0·55 (11)	5·00 ± 0·025 (11)
V_{null} (mV)	+45·7 ± 4·3 (14)	+49·5 ± 2·1 (11)	+40·3 ± 4·6 (11)
From tail current amplitude at −50 mV			
$V_{50\ activation}$ (mV)	+14·2 ± 1·8* (14)	+7·5 ± 2·6 (11)	+8·76 ± 2·72 (11)
k (slope factor)	9·32 ± 0·51 (14)	9·74 ± 0·91 (11)	9·86 ± 0·89 (11)
I_{max} (pA pF^{-1})	−61·0 ± 6·6* (14)	−96·4 ± 15·2 (11)	−75·7 ± 8·2 (11)
From steady state inactivation			
$V_{50,\ inact}$ (mV)	−31·8 ± 4·9 (5)	−33·1 ± 3·0 (4)	−35·7 ± 11·7 (4)
k (slope factor)	13·0 ± 1·5 (5)	12·9 ± 1·8 (4)	8·72 ± 1·20 (4)

The parameters were obtained as described in the legend to Fig. 2. Results here and in the text are given as means ± S.E.M. with the number of experiments in parentheses.* $2P < 0·05$ *vs.* nonsense (Student's *t* test).

measured, and the voltage dependence of activation of I_{Ba} was shifted about 7 mV in the depolarizing direction, following β-subunit antisense oligonucleotide injection (Fig. 5; Table 1). This effect was observed both from the $I-V$ curve, and from the voltage dependence of the tail current amplitude recorded at −50 mV (Fig. 5; Table 1). Representative examples are shown of current–voltage relationships, tail current activation and traces of I_{Ba} from cells injected with nonsense (Fig. 5*A* and *C*) or β-antisense oligonucleotides (Fig. 5*C* and *D*). The second major effect was a reduction by 46·6% of the peak amplitude of the maximum current (at the voltage determined from the $I-V$ relationships; Table 1). The maximum tail current amplitude following repolarization to −50 mV was also reduced by 36·7% (Table 1). There was no effect on the kinetics of activation of the maximum current (Table 1) although, at the same test potential, currents in the β-antisense-injected cells activated more slowly (Fig. 5*B* and *C*) because of the shift in the voltage dependence of activation. Similarly, the maximum calcium channel current in β-antisense-injected cells inactivated faster, possibly because of the voltage dependence of inactivation. No change was seen in the apparent reversal potential of the current, nor in the steady-state inactivation parameters (Table 1). In control experiments, no differences were observed in I_{Ba} between cells injected with nonsense oligonucelotide for 108 h and control non-injected cells of the same age (Table 1).

Effect of depletion of β-subunit immunoreactivity on the response to the DHP agonist Bay K 8644

In studies where the β-subunit was co-expressed with α_1-subunits, an increase in the number of DHP binding sites was observed (Varadi *et al.* 1991; Perez-Reyes *et al.* 1992), and variable effects on the response to the DHP agonist Bay K 8644 (Varadi *et al.* 1991; Itagaki *et al.* 1992; Perez-Reyes *et al.* 1992). For this reason, we examined the

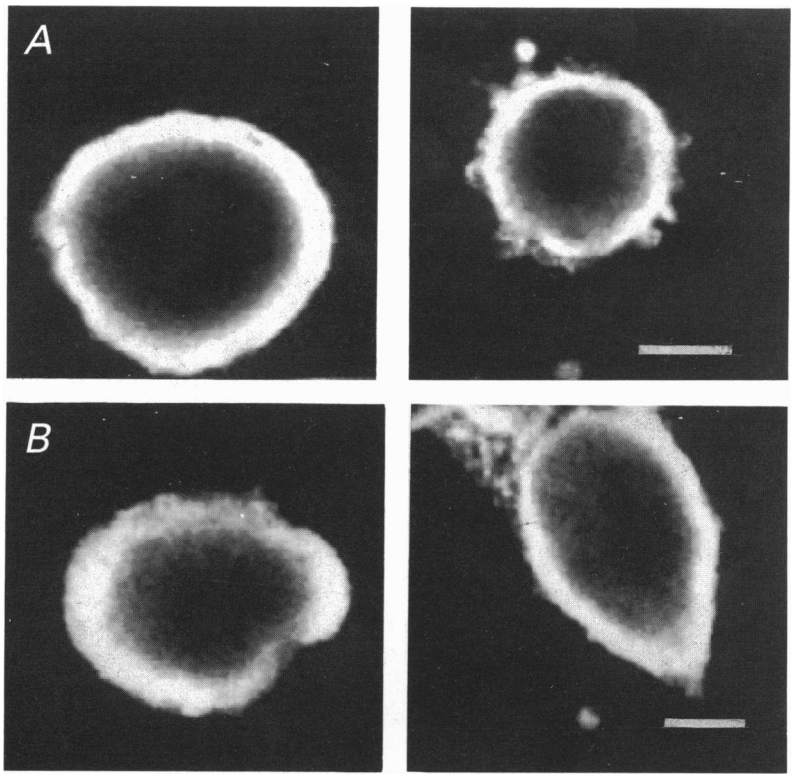

Figure 4. The lack of effect of VDCC β-antisense oligonucleotide injection on immunostaining for $G\alpha_o$ or VDCC α_2

A, immunostaining for G protein $G\alpha_o$ was performed as described in Campbell *et al.* (1993) (except that affinity-purified antibody was used), before (left) and 108 h after (right) microinjection of β-subunit antisense oligonucleotide. No reduction in staining for $G\alpha_o$ was observed. *B*, immunostaining for VDCC α_2 using an anti-peptide antibody (see Methods). Characterization of this antibody will be described in detail elsewhere (K. Brickley, V. Campbell, N. Berrow, D. Wray, R. Leach, R. I. Norman, S. Baldwin and A. C. Dolphin, unpublished observations). No reduction in staining for VDCC α_2 was observed 108 h following β-antisense oligonucleotide injection (right) compared with control (left).

effect of Bay K 8644 in the present system. Racemic Bay K 8644 (1 μM) was applied after determining an initial current–voltage relationship. A further current–voltage relationship was measured in its presence. Bay K 8644 increased the maximum current by 25·2% from 39·3 ± 5·7 to 49·2 ± 5·0 pA pF^{-1} ($n = 11$; $P < 0.05$) in a pooled group of control and nonsense-oligonucleotide-injected cells (Fig. 6*A*). In contrast, a small decrease in the maximum current was observed in β-subunit-antisense-injected cells by 13% from 28·0 ± 5·7 to 24·8 ± 4·9 pA pF^{-1} ($n = 10$), although the current–voltage relationship was still shifted to more hyperpolarized potentials by Bay K 8644 (Fig. 6*B*). Note that in this subgroup of cells, injection of the β-subunit antisense oligonucleotide resulted in a reduction in the control current of 28·8%, but no significant shift in the voltage for current activation was seen, possibly because of the smaller number of cells used in the averaged current–voltage relationships (Fig. 6).

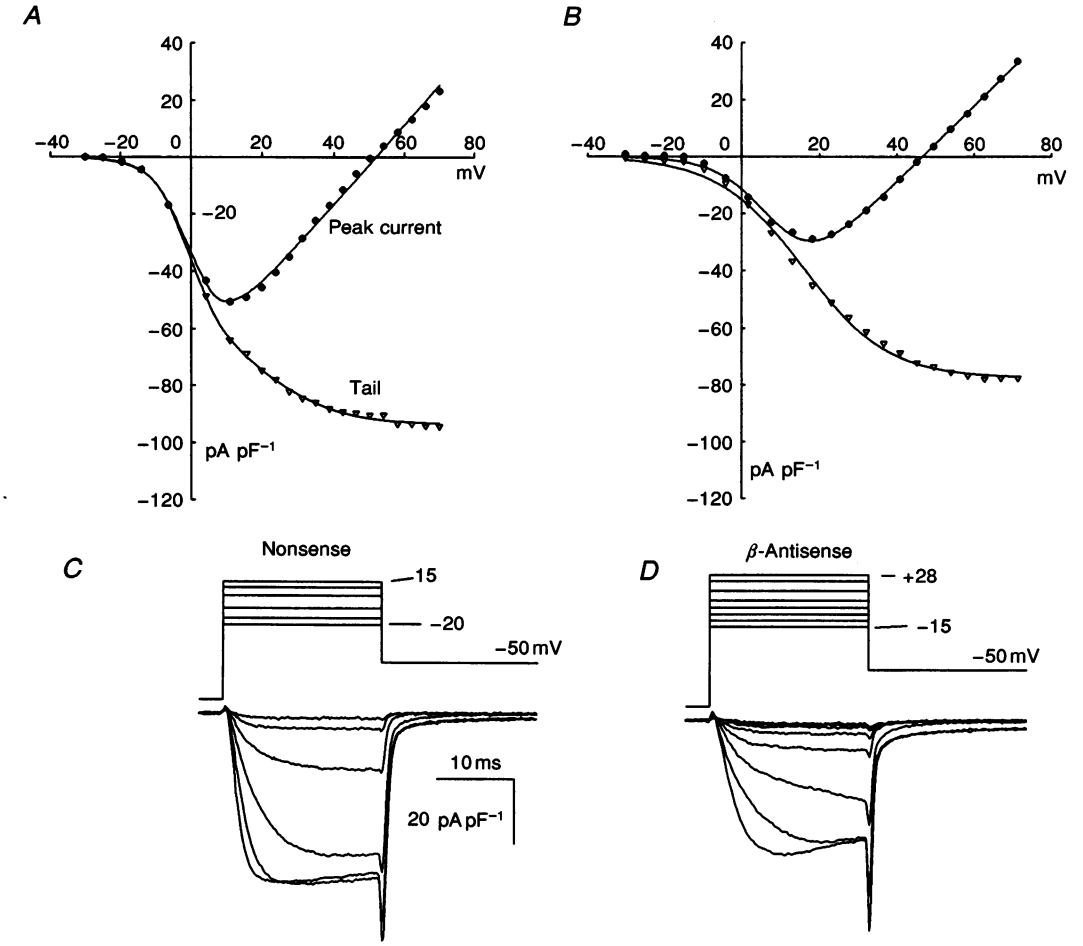

Figure 5. Effect of depletion of VDCC β-subunit immunoreactivity on amplitude and voltage-dependence of activation of I_{Ba} in DRGs

A and *B*, cells were injected with VDCC β-subunit, or nonsense, oligonucleotide 108–116 h previously. I_{Ba} was recorded from spherical neurones with a diameter of 10–15 μm and a capacitance of less than 30 pF. Current–voltage relationships were performed and the peak current recorded during 20 ms steps to test potentials from −30 to +70 or 80 mV. Representative current–voltage relationships are shown (●). The tail current amplitudes were determined following repolarization to −50 mV (▽). The current–voltage relationship was fitted to a Boltzmann equation of the form:

$$I = g(V - V_{null})/\{1 + \exp[(V - V_{50})/k]\},$$

where V_{null} is the null potential at which no current flows, V_{50} is the voltage for 50% activation and k is the slope factor. The tail current activation was fitted to a Boltzmann relationship of the form:

$$I = I_{max}/\{1 + \exp[(V - V_{50})/k]\},$$

where I_{max} is the maximum current in pA pF^{-1}. The fits are given by the continuous lines. *C* and *D*, current traces are shown for the activation of a nonsense- and β-antisense-injected cell respectively.

DISCUSSION

The results of the present study indicate that it is possible to deplete DRGs of VDCC β-subunit immunoreactivity, by injection of an antisense oligonucleotide with a high complementarity to all four VDCC β-subunit mRNAs. The antipeptide antiserum used in these experiments was raised to a sequence common to β_1, β_2 and β_4, and it cross-reacted with the corresponding β_3 peptide sequence. It recognized a protein of approximate mol. mass of 52 kDa in rat skeletal muscle T-tubules, and a protein of 55 kDa in a purified DHP receptor from rabbit skeletal muscle (probably β_{1a}). The immunoreactivity shown in rat skeletal muscle sections is consistent with the recognition by the antiserum of VDCC β-subunits present in high concentration in T-tubules. Two protein bands were recognized in rat brain membranes by the β-subunit antiserum under different conditions, with mol. mass of approximately 52 and 65 kDa, similar to the mol. mass (calculated from cDNA) of the rat brain β_3- (54·6 kDa) and β_2- (68·2 kDa) or β_{1b}- (65·7 kDa) subunits, respectively (Pragnell *et al.* 1991; Perez-Reyes *et al.* 1992; Castellano *et al.* 1993*b*). In cultured rat DRGs the β-subunit immunoreactivity is cytoplasmic, with a concentration at the inner surface of the plasma membrane consistent with its association with VDCCs. In view of the cytoplasmic staining, it is possible that β-subunits have other functions in neurones in addition to their role in VDCCs.

The time course of reduction of VDCC β-subunit immunoreactivity following microinjection of the β-subunit antisense oligonucleotide showed maximal depletion at between 108 and 130 h after injection, giving an estimate of the half-life of β-subunits of about 55–60 h. This is in contrast to the much more rapid turnover of the α-subunit of G_o, also a membrane-associated protein. A similar rate of turnover of $G\alpha_o$ was previously observed by Kleuss *et al.* (1991). The finding that recovery of the levels of both proteins began at 144 h after oligonucleotide injection indicates that this is the limit of effectiveness of a single phosphorothioate oligonucleotide injection in this system.

The effects on I_{Ba} of β-subunit oligonucleotide injection are consistent with prior results from co-expression studies and indicate an important role for the VDCC β-subunits in regulating the biophysical properties of expressed

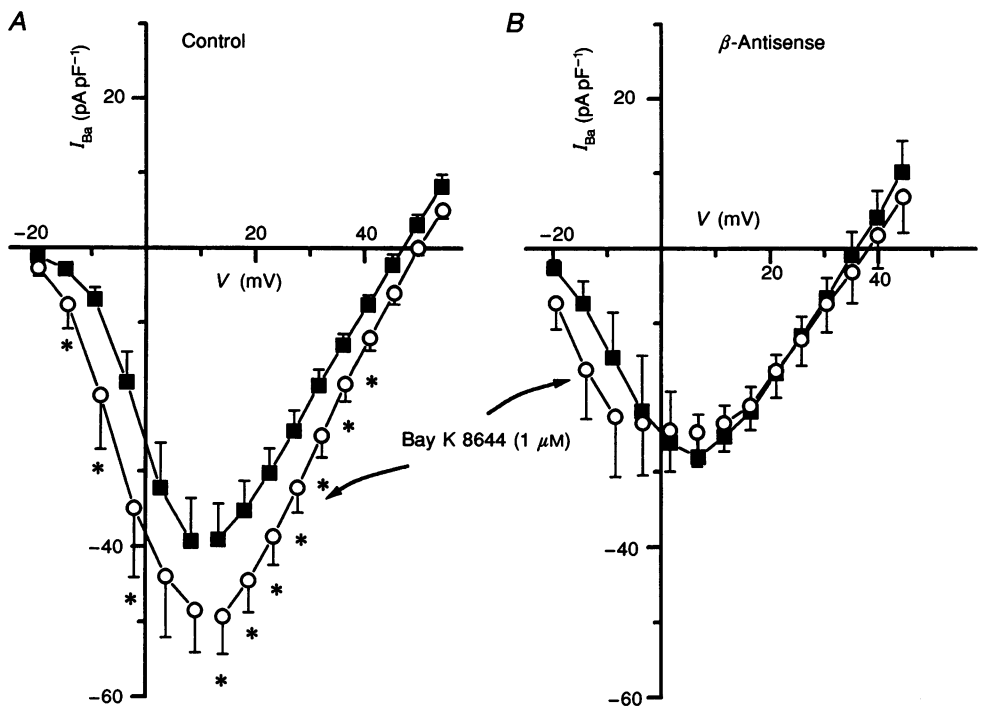

Figure 6. The effect of Bay K 8644 on I_{Ba} in control and VDCC β-subunit antisense-injected cells

A, mean current–voltage relationships from 7 control and 4 nonsense-injected (108–116 h previously) cells showing an increase in mean I_{Ba} following Bay K 8644 (1 μM) application (○) compared with prior to application (■). The increase was seen in both control and nonsense-injected cells and for this reason these data have been pooled. *B*, mean current–voltage relationships from 10 β-subunit antisense-injected (108–116 h previously) cells showing no enhancement in the current following Bay K 8644 except a non-significant shift at negative clamp potentials. These results also confirm the reduction in the control current in *B* (■) compared with that in *A*. * $P < 0{\cdot}05$ compared with control (Student's *t* test).

α_1-subunits. Loss of β-subunit immunoreactivity resulted in a reduction in I_{Ba} and a depolarizing shift in the current–voltage relationship, the opposite of results obtained on co-expression of β- with α_1-subunits. This has been shown for skeletal muscle, cardiac and neuronal (α_{1B} and α_{1A}) calcium channels (Varad *et al.* 1991; Hullin *et al.* 1992; Castellano *et al.* 1993*a*, *b*; Zhang *et al.* 1993).

In a number of co-expression studies it has been shown that the expression of β-subunits increased the number of DHP receptors, although it has also been found that this is not due to increased expression of the α_1-subunit protein (Neely, Wei, Olcese, Birnbaumer & Stefani, 1993; Nishimura, Takeshima, Hofmann, Flockerzi & Imoto, 1993). It is possible that the β-subunit regulates the equilibrium between two DHP binding states of α_1, the higher affinity state being favoured by the presence of the β-subunit. This is supported by the present finding that Bay K 8644 was less effective following β-antisense oligonucleotide injection although it should be noted that racemic Bay K 8644 was used, and only the (−)-isomer has agonist properties. It will be of interest to examine the effect of DHP antagonists following VDCC β-subunit depletion. However, co-expression of β- with α_1-subunits in oocytes has generally resulted in a reduction of sensitivity to DHPs (Varadi *et al.* 1991; Hullin *et al.* 1992; Itagaki *et al.* 1992), which may result from enhancement by β of the endogenous DHP-insensitive calcium current of the oocyte. In other studies no change of sensitivity to DHPs was seen (Perez-Reyes *et al.* 1992). An alternative explanation for the present results is that current through L-type VDCCs is preferentially inhibited by the β-subunit depletion strategy used. Some evidence suggests that neuronal L-type channels are associated with β_{1b} whereas N-type channels are associated with β_3 (Witcher *et al.* 1993). It is possible, but unlikely, that we have produced a selective depletion of β_{1b} in the present experiments. This conclusion is supported by the finding that ω-CTX produced a similar inhibition following β-subunit antisense injection as in control cells (V. Campbell, N. S. Berrow, E. M. Fitzgerald, R. Wade & A. C. Dolphin, unpublished observations), indicating that the residual current does not exhibit a greater proportion of N-current.

In conclusion, our results provide evidence that the high threshold VDCCs native to DRGs are associated with β-subunits which strongly influence their biophysical properties and pharmacology. In further experiments to examine the ability of the $GABA_B$ agonist (−)-baclofen to modulate calcium channel currents following depletion of β-subunit immunoreactivity, we have found an enhanced response to agonist of the residual current, suggesting that the β-subunit is also involved in calcium current modulation (V. Campbell, N. S. Berrow, E. M. Fitzgerald, R. Wade & A. C. Dolphin, unpublished observations).

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