Voltage-dependent binding and calcium channel current inhibition by an anti-α_{1D} subunit antibody in rat dorsal root ganglion neurones and guinea-pig myocytes

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1. The presence of calcium channel α_{1D} subunit mRNA in cultured rat dorsal root ganglion (DRG) neurones and guinea-pig cardiac myocytes was demonstrated using the reverse transcriptase-polymerase chain reaction.

2. An antipeptide antibody targeted at a region of the voltage-dependent calcium channel α_{1D} subunit C-terminal to the pore-forming SS1–SS2 loop in domain IV (amino acids 1417–1434) only bound to this exofacial epitope if the DRG neurones and cardiac myocytes were depolarized with 30 mM K^+.

3. Incubation of cells under depolarizing conditions for 2–4 h with the antibody resulted in a maximal inhibition of inward current density of 49% ($P < 0.005$) for DRGs and 30% ($P < 0.05$) for cardiac myocytes when compared with controls.

4. *S*-(−)-Bay K 8644 (1 μM) enhanced calcium channel currents in DRGs by 75 ± 19% ($n = 5$) in neurones incubated under depolarizing conditions with antibody that had been pre-adsorbed with its immunizing peptide (100 μg ml^{-1}). This was significantly ($P < 0.05$) larger than the enhancement by *S*-(−)-Bay K 8644 that was seen with cells incubated under identical conditions but with antibody alone, which was 15 ± 4% ($n = 5$).

5. These results demonstrate the presence of calcium channel α_{1D} subunits in rat DRG neurones and guinea-pig cardiac myocytes. They also show that amino acids 1417–1434 of the α_{1D} subunit are only exposed to the extracellular face of the membrane following depolarization and that the binding of an antibody to these amino acids attenuates calcium channel current and reduces the ability of *S*-(−)-Bay K 8644 to enhance this current, indicating that it is an L-type current that is attenuated.

The voltage-dependent calcium channels (VDCCs) are a group of membrane-spanning hetero-oligomeric proteins that play a vital role in the function of excitable cells (for review see Dolphin, 1995). Using biophysical and pharmacological parameters, native calcium currents can be divided into several distinct components termed L, N, P, Q, R and T (Birnbaumer *et al.* 1994). In recent years, cloning techniques have demonstrated the existence of six distinct pore-forming α_1-subunits, which combine with several accessory subunits to give rise to the calcium channel family. These α_1-subunits are called A, B, C, D, E and S (for review see Dolphin, 1995). Of these six, C, D and S are involved in the formation of the L-type calcium channels, class C being found in smooth muscle, cardiac muscle and neurones; class D particularly in neurosecretory cells; and class S in skeletal muscle.

VDCC α_{1D} subunits have been detected in a variety of cell types in central neurones (Hell *et al.* 1993) and have been shown to be particularly abundant in brain areas responsible for neuroendocrine function, such as the hypothalamus and the pituitary gland (Chin, Smith, Kim & Kim, 1992). It is, therefore, likely that α_{1D} subunits may play a fundamental role in excitation–secretion coupling and in the modulation of neuroendocrine functions. In addition, it has also been

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demonstrated that the release of substance P from dorsal root ganglion (DRG) neurones is sensitive to modulation by 1,4-dihydropyridines (DHPs) (Holz, Kream, Speigel & Dunlap, 1989) and therefore it is possible that the L-type channel-forming α_{1D} subunits are important in the DRG neurosecretory process.

It is characteristic of the L-type VDCCs that their electrophysiological properties can be selectively modulated using DHPs. This group of compounds may act either as calcium channel antagonists, e.g. nicardipine, nifedipine and (+)-isradipine, or as calcium channel agonists, e.g. *S*-(−)-Bay K 8644. Indeed, the ability of certain of these compounds to inhibit calcium channels has been advantageous in the development of antihypertensive, antiarrhythmic and antianginal drugs (Janis & Triggle, 1991).

A considerable quantity of data has been amassed regarding the molecular mechanism of action of DHPs. Photoaffinity labelling studies of calcium channels located in their native membranes (as opposed to the solubilized protein) demonstrated that two distinct sequence regions were involved in the binding of DHPs. These were located at the extracellular surface in the IIIS5–IIIS6 linker and at the extracellular end of segment IVS6 (Nakayama, Taki, Streissnig, Glossmann, Catterall & Kanaoka, 1991; Streissnig, Murphy & Catterall, 1991; Catterall & Streissnig, 1992). However, it has also been demonstrated that DHPs bind to a cytosolic region of segment IVS6 (Regulla, Schneider, Nastainczyk, Meyer & Hofman, 1991). This was in contrast to the binding sites predicted by several electrophysiological studies, which indicated that the DHP-binding region(s) were located on or close to the extracellular surface of the calcium channel (Kass, Arena & Chin, 1991; Strubing, Hering & Glossmann, 1993). More recently, experiments using chimeric α_1-subunits showed that the DHP sensitivity of a functionally expressed α_{1C} subunit (Mikami *et al.* 1989) could be abolished if certain corresponding regions of an α_{1A} subunit (Mori *et al.* 1991) were substituted (Tang, Yatani, Bahinski, Mori & Schwartz, 1993). The linker region between IVS5 and IVS6 appeared to be a major site for DHP action. Further studies (Grabner, Wang, Hering, Streissnig & Glossmann, 1996) demonstrated that the minimum α_{1C} sequence stretches that conferred DHP sensitivity to an α_{1A} subunit consisted of regions of IIIS5, IIIS6 and their connecting linker, and in addition that the IVS5–IVS6 linker and a segment of IVS6 were required. Differences between agonist and antagonist action appeared to centre on binding differences within repeat IV. The importance of repeat IV in DHP action was further reinforced when Schuster, Lacinova, Klugbauer, Ito, Birnbaumer & Hofmann (1996) showed that mutation of three amino acids, Tyr1485, Met1486 and Ile1493, in segment IVS6 of an α_{1C} subunit reduced the affinity of the DHP antagonist (+)-isradipine 100-fold and abolished the stimulatory action of *S*-(−)-Bay K 8644.

In this study, the presence of a DHP-sensitive α_{1D} subunit in both cultured rat DRG neurones and guinea-pig cardiac myocytes has been demonstrated using the reverse transcriptase-polymerase chain reaction (RT-PCR). An α_{1D} subunit-specific antibody targeted to amino acids 1417–1434 of the rat brain α_{1D} clone, a sequence corresponding to part of a region reported to confer DHP agonist sensitivity (Grabner *et al.* 1996), was raised. The effect of this antibody on calcium channel currents in DRGs and cardiac myocytes and its effect on DHP sensitivity were also investigated.

Preliminary accounts of these findings have appeared previously (Campbell *et al.* 1995*b*; Wyatt, Brickley & Dolphin, 1996).

METHODS

Tissue culture

Dorsal root ganglia were dissected from 1- to 2-day-old Sprague–Dawley rats killed by decapitation. The ganglia were incubated with collagenase (1·25 μg ml^{-1}; Sigma) 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}). They were then triturated with a fire-polished Pasteur pipette in the presence of DNase (1600 Kunitz ml^{-1}). DRG neurones were plated on polyornithine–laminin-coated glass coverslips at an approximate density of 1×10^4–3×10^4 cells (2·2 cm)$^{-2}$ in the above medium supplemented with nerve growth factor (10 ng ml^{-1}; Sigma). After 24 h in culture at 37 °C in air containing 5% CO_2, the neurones were incubated with cytosine-arabino-furanoside (5 ng ml^{-1}) for 24 h. The medium was changed every 3–4 days. Cells were used for electrophysiological recording and immunocytochemistry after 7–12 days in culture.

To isolate ventricular myocytes, male guinea-pigs (350–650 g) were heparinized (200–500 u, I.P.) and following cervical dislocation the heart was removed and placed in Krebs–Henseleit (K–H) solution containing (mM): NaCl, 119; KCl, 4·2; $CaCl_2$, 1·0; $MgSO_4$, 0·94; KH_2PO_4, 1·2; $NaHCO_3$, 25; glucose, 11·5; equilibrated with 95% O_2–5% CO_2 at 35 °C giving a pH of 7·4. The aorta was cannulated and the heart retrogradely perfused on a Langendorff apparatus with fresh K–H solution for 5 min. The solution was then replaced with a modified low-Ca^{2+} Tyrode solution containing (mM): NaCl, 120; KCl, 5·4; $MgSO_4$, 5·0; pyruvate, 5·0; glucose, 20; taurine, 20; Hepes, 10; nitrilotriacetic acid (NTA), 5·0; pH 7·0 at 35 °C. $CaCl_2$ was added to give a free [Ca^{2+}] of approximately 12 μM. After another 5 min, the perfusion solution was again replaced with a low-Ca^{2+} Tyrode solution, this time without NTA and containing 200 μM free Ca^{2+} and 4 units ml^{-1} protease (Type XXIV; Sigma). After this solution had been perfused through the heart for 1 min, the protease-containing solution was replaced with another modified Tyrode solution containing 200 μM Ca^{2+} and 1 mg ml^{-1} collagenase (Lorne Laboratories) and 0·6 mg ml^{-1} hyaluronidase (Sigma). This was perfused through the heart for 10 min. The heart was then removed from the Langendorff apparatus, and the ventricles chopped and gently shaken for two periods of 5 min in fresh low-Ca^{2+} Tyrode solution (200 μM free Ca^{2+}) containing 1·0 mg ml^{-1} collagenase and 0·6 mg ml^{-1} hyaluronidase at 35 °C in an atmosphere of 100% O_2. The dissociated cells were then strained from the other material through 300 μm nylon gauze and centrifuged at low speed for 30 s to 1 min. The pellet produced was then resuspended and kept in K–H solution at room temperature (21–24 °C) before use. Cells were allowed to settle onto coverslips coated with laminin (Life Technologies, Paisley, UK) for 5 min

before being used for electrophysiological or immunocytochemical experiments.

RT-PCR on DRGs and cardiac myocytes

Total RNA was prepared from DRGs and purified guinea-pig cardiac myocytes by the guanidinium thiocyanate method of Chomczynski & Sacchi (1987) and purified by extraction with phenol (pH < 5) and chloroform. Residual genomic DNA was digested with RQ DNase (5 U; Promega, Madison, WI, USA) for 15 min at 37 °C and the reactions were terminated by extraction with phenol. Reverse transcription was carried out using 200 units M-MLV RT (Promega) in the supplied buffer, 20 units RNasin (Promega), 1·25 mM of each deoxyribonucleoside triphosphate (dNTP) and random hexamer primers (Promega), in a total volume of 20 μl at 37 °C for 60 min. The enzyme was inactivated by incubation at 80 °C for 5 min. The resulting cDNA was amplified by PCR using primers enclosing the α_{1D} antibody-binding site. The sense primer, Da1SE (5′-TTAGTGACGCCTGGAACACG-3′) was targeted to an α_{1D} sequence that is conserved between human (Genbank accession numbers: M83566, M76558), rat (M57682), mouse (M57975) and hamster (M57969, M57970). The antisense primer, ConR (5′-CCTGTATCAGGAAAGTGG-3′) is conserved in α_{1D} and α_{1C} subunits between human (L04569), rat (M67515), mouse (L01776) and rabbit (X55539). PCR was carried out in a volume of 50 μl containing 2 units BioTaq™ (Bioline Ltd, London) in the supplied buffer supplemented with 1·5 mM $MgCl_2$, 200 μM of each dNTP and 0·4 μM of each primer. The following cycles were used: 95 °C for 3 min (1 cycle), 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (30 cycles), 72 °C for 4 min (1 cycle). The sequence of the PCR product was confirmed as α_{1D} using the SequiTherm™ Cycle Sequencing kit (Epicenter Technologies, Madison, WI, USA) with an α_{1D} primer, Pr3SE (5′-GGTTGCCATGAGAGATAACA ACC-3′) located close (see Fig. 1*A*) to the antibody-binding site.

Anti-α_{1D} and -α_{1C} antisera

The α_{1D} subunit antiserum used in this study was raised against a peptide corresponding to amino acids 1417–1434 of the rat neuronal L-type α_{1D} subunit (Hui, Ellinor, Krizanova, Wang, Diebold & Schwartz, 1991). This peptide sequence (KLCDPD SDYNPGEEYTC) is specific, in rat brain, for the class D polypeptide but is also present in the skeletal muscle calcium channel α_{1S} subunit with one amino acid change (KLCDPDSDYAPG EEYTC). However, no related peptide sequence is present in the α_{1C} subunit. The α_{1C} antibody used was raised against an epitope in the intracellular region IIS6–IIIS1 corresponding to amino acids 797–812 (EESKEEKIELKSITAD) of the rat brain α_{1C} subunit (Snutch, Tomlinson, Leonard & Gilbert, 1991). Polyclonal antisera to these peptides were generated in rabbits using standard techniques (Brickley *et al.* 1995) and recognized the immunizing peptides in enzyme-linked immunosorbent assays (data not shown). The α_{1D} antibody also inhibited DHP-sensitive calcium entry in rat B lymphocytes (Akha, Willmott, Brickley, Dolphin, Galione & Hunt, 1996).

Immunocytochemistry

Both cultured DRG neurones and ventricular myocytes (that had been allowed to settle on coverslips for 5 min) were washed with 150 mM NaCl containing 40 mM Tris pH 7·4 (TBS), and incubated for 2 min at 37 °C in a K–H solution containing either 4·2 or 30 mM KCl in order to depolarize the cells; these solutions had no

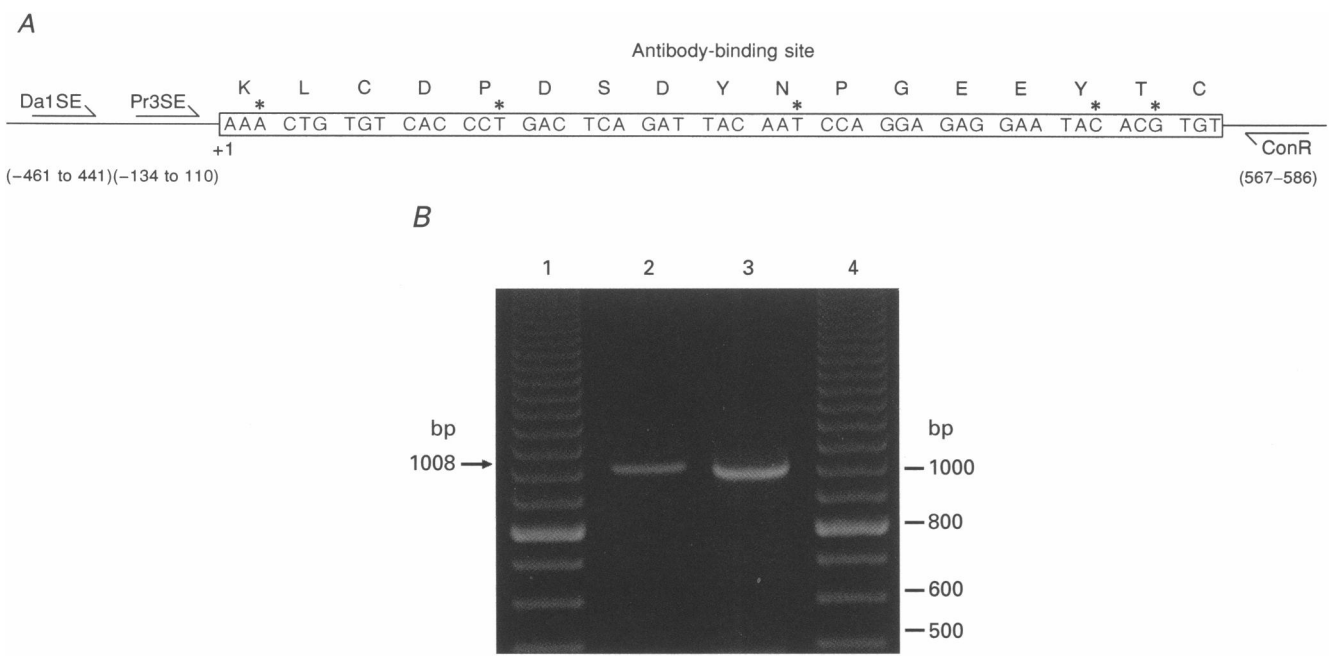

Figure 1. Verification of the presence of VDCC α_{1D} subunits in cultured rat DRG neurones and isolated cardiac myocytes

A shows primer and antibody-binding sites. Numbers in parentheses refer to nucleotides relative to the first nucleotide of the sequence coding for the antibody-binding site, which is nucleotide 4778 of the rat brain D-type calcium channel α_1-subunit (M57682). Asterisks indicate five silent nucleotide exchanges between sequences of rat and guinea-pig; all are in the 3rd position of the codon. *B*, lanes 2 and 3 show amplification products of the primer couple Da1SE–ConR containing the sequence coding for the antibody-binding site. In lane 2, cDNA of purified guinea-pig cardiac myocytes was used as a template. In lane 3, the template was cDNA obtained from DRG neurones. Lanes 1 and 4 contain a 100 bp ladder (Pharmacia).

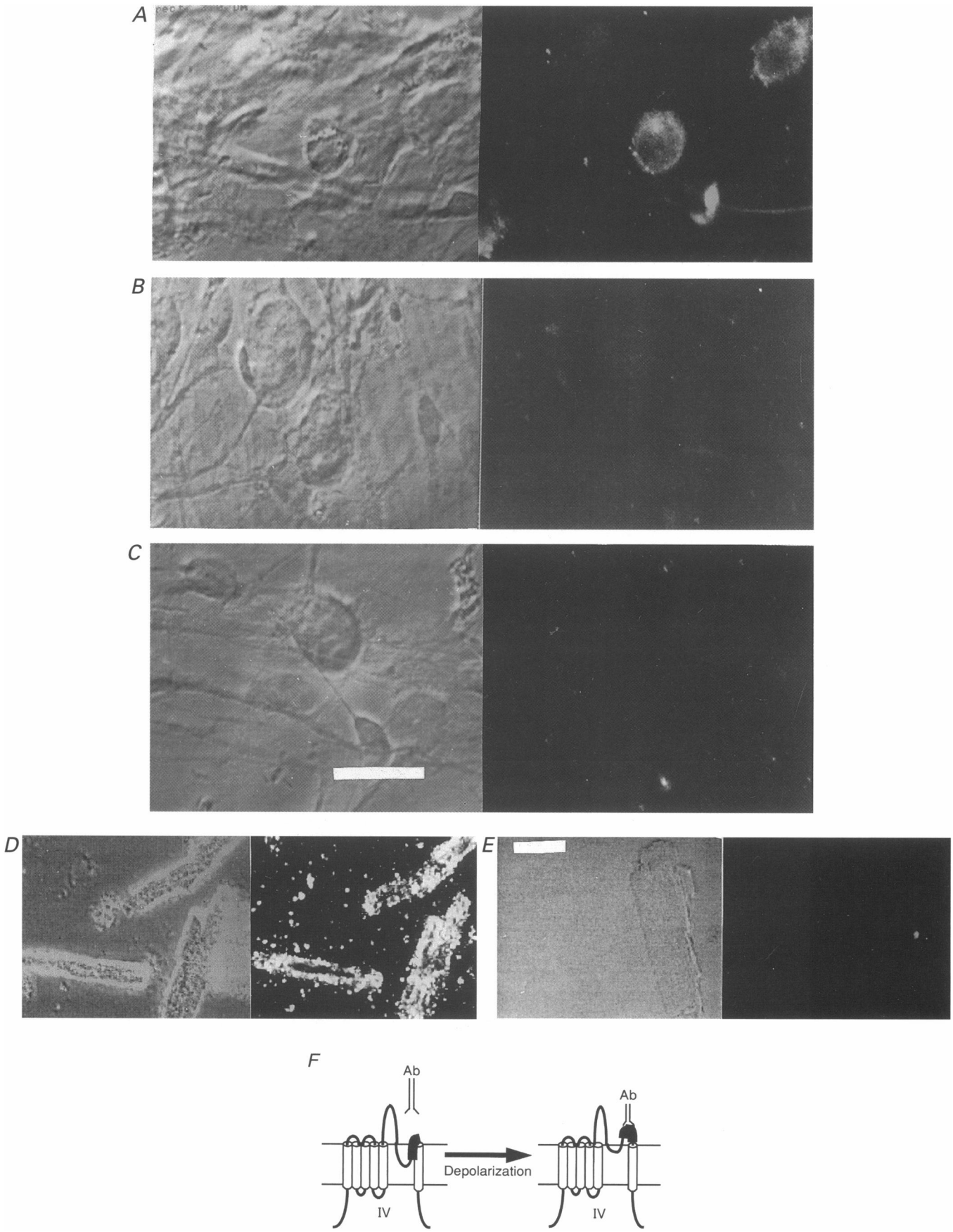

Figure 2. For legend see facing page.

added Ca^{2+}. The cells were then fixed with 4% paraformaldehyde in TBS for 30 min at room temperature. Permeabilization of the cells, when required, was achieved by 3 × 5 min washes in 0·02% (v/v) Triton X-100 in TBS. Cells were then washed (3 × 5 min) with TBS containing 20% goat serum (Sigma), 4% bovine serum albumin and 0·1% DL-lysine and incubated with a 1 : 1000 dilution of anti-α_{1D} subunit antiserum or preimmune serum overnight at 4 °C. In experiments in which the antibody was pre-adsorbed with its immunizing peptide, the antibody was pre-incubated with the peptide (100 μg ml^{-1}) for 30 min at 37 °C before being applied to the cells. The cells were then washed again in TBS–serum mixture (4 × 5 min) and incubated with goat anti-rabbit IgG conjugated to biotin (1: 200 dilution; Sigma) for 2 h at 4 °C before being washed in TBS–serum mixture (4 × 5 min) and incubated for 1 h at room temperature with extravidin conjugated to fluorescein (1 : 50 dilution; Sigma). Cells were finally washed (5 × 5 min) with TBS and mounted in antifade mountant (Citifluor, City University, London) before being viewed with a confocal scanning laser microscope (MRC-600; Bio-Rad). All images were recorded with constant data aquisition parameters for each experiment.

Experiments were also carried out to investigate whether calcium channel internalization occurred during the period of incubation with the α_{1D} antibody (for electrophysiology experiments see next section). To elucidate this, DRG neurones were incubated under depolarizing conditions (30 mM KCl K–H solution, Ca^{2+} free) for 2 h at room temperature in the presence of α_{1D} antiserum (1 : 50 dilution, as for electrophysiology experiments) before being fixed as before. Neurones were then permeabilized, or not, as described previously and the above staining technique repeated. If the neurones had been previously exposed to the α_{1D} antibody the procedure was begun at the wash stage before application of the secondary antibody.

COS-7 cells (an African green monkey kidney fibroblast cell line) were transiently transfected by electroporation as described previously (Campbell, Berrow, Brickley, Page, Wade & Dolphin, 1995*a*) with full length constructs of α_{1C} (15 μg; Snutch *et al.* 1991) or α_{1D} (15 μg; Hui *et al.* 1991), $\alpha_2\delta$ (10 μg; Kim, Kim, Lee, King & Chin, 1992) and β_{1b} (5 μg; Pragnell, Sakamoto, Jay & Campbell, 1991). All cDNAs were subcloned by standard molecular biological methods into pMT-2.

After 3 days the cells were used for immunocytochemistry; the method for COS-7 cells was essentially the same as for DRGs except that the cells were not incubated in K–H solution prior to fixing. The fixing time was reduced to 15 min after which the cells were permeabilized with 0·02% Triton X-100 for 15 min. The α_{1D} and α_{1C} antisera were diluted by 1 : 500 and the fluorophore used was Texas Red (1 : 100).

Electrophysiology of DRG neurones

Recordings were made with a patch pipette solution containing (mM): caesium aspartate, 140; EGTA, 5·0; $MgCl_2$, 2·0; $CaCl_2$, 0·1; K_2ATP, 2·0; GTP, 0·1; Hepes 10; pH 7·2, and adjusted to 310 mosmol l^{-1} with sucrose. The extracellular solution contained (mM): TEA-Br, 160; KCl, 3; $NaHCO_3$, 1·0; $MgCl_2$, 1·0; Hepes, 10; glucose, 4; $BaCl_2$, 1·0; tetrodotoxin, 0·0005; pH 7·4, and adjusted to 320 mosmol l^{-1} with sucrose. Patch pipettes (2–5 MΩ) were made from borosilicate glass, fire polished and coated with Sigmacote® (Sigma). Inward currents were evoked from a holding potential of −80 mV by stepping to voltages between −40 and 50 mV in 5 mV steps. An Axopatch 200A or -1D amplifier (Axon Instruments) was used; recordings were filtered at 2 kHz (4-pole low-pass Bessel filter) and digitized at 10–50 kHz using a Digidata 1200 A/D converter. Currents were analysed using pCLAMP 6.0.2 (Axon Instruments) and leak subtracted using a *P*/6 protocol. The holding current at −80 mV was usually less than 30 pA; series resistance was normally less than 10 MΩ and was routinely compensated by at least 75%.

S-(−)-Bay K 8644 was obtained from Bayer (Germany) and nicardipine from Sigma. They were applied by low pressure ejection onto the cells from a pipette located approximately 30 μm from the cell. All experiments using these compounds were carried out at low light intensity.

Prior to conventional whole-cell patch clamp recordings being made, the DRG cells were incubated for 2–4 h at room temperature with α_{1D} antibody (1 : 50 dilution) or α_{1D} antibody that had been pre-incubated with its immunizing peptide (100 μg ml^{-1}) under depolarizing or non-depolarizing conditions (30 mM KCl K–H solution or conventional K–H solution, respectively).

Electrophysiology of cardiac myocytes

Myocytes were incubated with the antibody as described above, then superfused with normal Tyrode solution containing (mM): NaCl, 140; KCl, 6; $CaCl_2$, 2·5; $MgCl_2$, 1; Hepes, 10; glucose, 10; pH 7·4. Impalement electrodes had resistances of 10–25 MΩ and contained (mM): KCl, 2000; EGTA, 0·1; Hepes, 5; pH 7·2. Voltage clamp experiments were carried out using an Axoclamp-2A amplifier in switch clamp mode (discontinuous single microelectrode voltage clamp), switching at 5 kHz at gains ⩾ 1 nA mV^{-1}. Calcium currents were evoked from a holding potential of −40 mV by depolarization to +50 mV in 10 mV steps. Voltage clamp software for data acquisition, analysis and control was kindly provided by John Dempster, University of Strathclyde, UK.

All data are given as means ± S.E.M. of the stated numbers of determinations (n). Statistical significance was determined by Student's t test.

Figure 2. Confocal immunocytochemical localization of VDCC α_{1D} subunit immunoreactivity in DRG neurones and cardiac myocytes

A, membrane immunostaining for α_{1D} in non-permeabilized cultured rat DRGs that were depolarized with 30 mM K^+ for 2 min and then fixed with paraformaldehyde. *B* demonstrates lack of immunostaining in non-permeabilized DRG neurones that were not depolarized before fixation. *C* shows lack of immunostaining in depolarized and fixed DRG neurones following incubation with antiserum pre-adsorbed with its immunizing peptide (100 μg ml^{-1}). Scale bar in *C* applies to *A* and *B* and represents 15 μm. *D*, membrane immunostaining for α_{1D} in non-permeabilized cardiac myocytes that were depolarized with 30 mM K^+ and then fixed with paraformaldehyde. *E* demonstrates lack of immunostaining in non-permeabilized cardiac myocytes that were not depolarized before fixation. Scale bar in *E* applies to *D* and represents 25 μm. In *A*–*E*, the phase image is shown to the left and the fluorescence confocal image to the right. *F*, schematic diagram showing a possible conformational change in the channel protein upon depolarization, allowing the antibody to bind. The antibody-binding site is indicated by the thickened region.

RESULTS

Evidence for the presence of the VDCC α_{1D} isoform in rat DRG neurones and isolated guinea-pig myocytes

Total RNA was extracted from rat DRG neurones and guinea-pig cardiac myocytes (see Methods). Following reverse transcription, the resulting cDNA was amplified by PCR using an α_{1D} sense primer (Da1SE) and an antisense primer (ConR) specific to a sequence conserved in α_{1D} and α_{1C} subunits (see Methods and Fig. 1*A*). The size of the amplified products was 1008 bp as predicted from the DNA sequence (Fig. 1*B*). The sequences of the PCR products containing the α_{1D} antibody-binding site sequence were determined using a second α_{1D}-selective primer, Pr3SE (see Methods and Fig. 1*A*). This confirmed the presence of an α_{1D} subunit in both DRG neurones and guinea-pig cardiac myocytes. A sequence comparison revealed five nucleotide

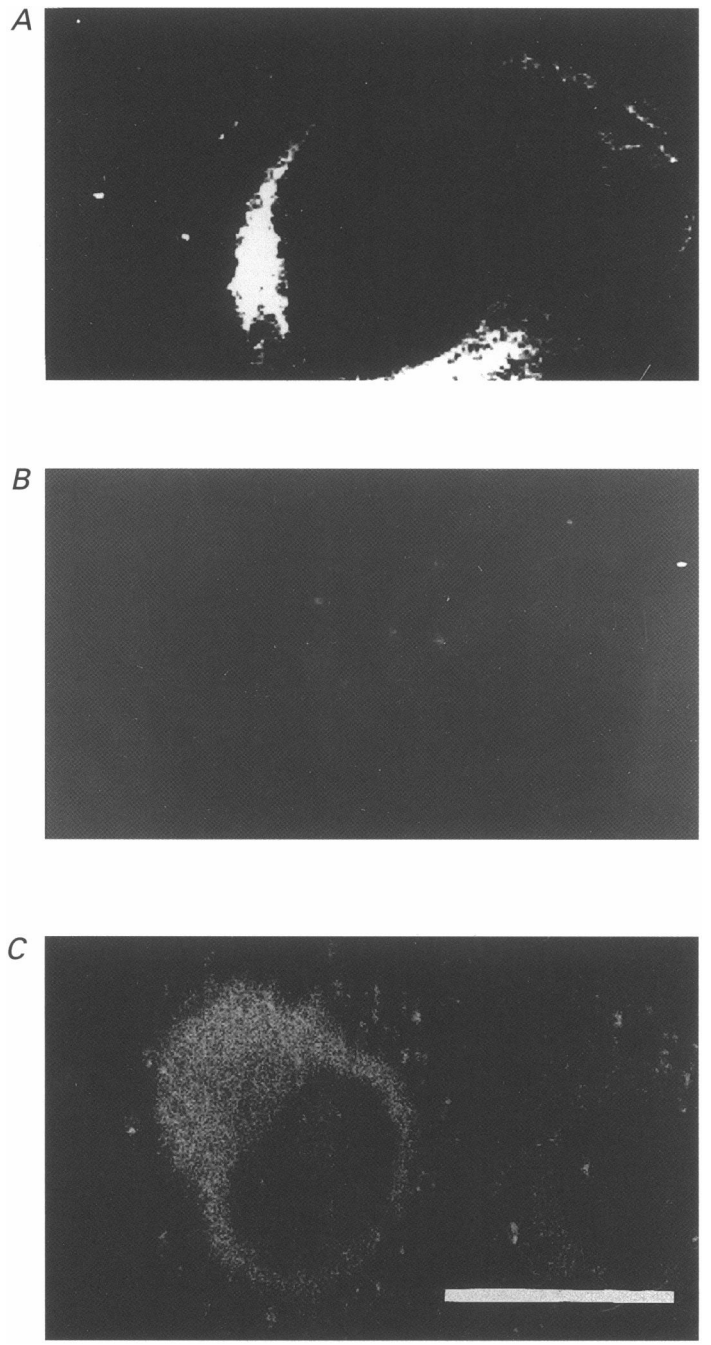

Figure 3. Antibody staining in COS-7 cells

A, α_{1C} staining in COS-7 cells transfected with cDNA encoding α_{1C}, $\alpha_2\delta$ and β_{1b} subunits. *B*, α_{1D} staining in COS-7 cells transfected with cDNA encoding α_{1C}, $\alpha_2\delta$ and β_{1b} subunits. *C*, α_{1D} staining in COS-7 cells transfected with cDNA encoding α_{1D}, $\alpha_2\delta$ and β_{1b} subunits. Scale bar in *C* applies to *A* and *B* and represents 20 μm.

exchanges in the guinea-pig sequence compared with the known rat brain α_{1D} sequence (M57682). All of the exchanges were silent and coded for the same amino acids.

Localization of the α_{1D} antibody-binding site

The anti-α_{1D} antiserum was raised against a peptide sequence located C-terminal to the SS1–SS2 pore-lining region on transmembrane domain IV of the α_1-subunit of the rat brain D class VDCC (Hui *et al.* 1991). This sequence differs by only one amino acid from that of the skeletal muscle VDCC α_{1S} subunit but is absent from all other cloned α_1-subunits. The antibody recognizes purified skeletal muscle DHP receptor on immunoblots (results not shown). The proposed membrane arrangement of the α_{1D} subunit suggested that the peptide sequence against which the α_{1D} antibody was raised was located on the exofacial surface of the plasma membrane. In both DRG neurones and cardiac myocytes no immunoreactivity was observed unless cells were permeabilized with Triton X-100, a detergent which solubilizes membrane lipids allowing the antibody to target its binding site within the membrane, after fixation ($n = 3$). However, cells that were depolarized with 30 mM K^+, before fixation, showed immunostaining in the absence of permeabilization, which was localized to the plasma membrane (Fig. 2*A* and *D*; $n = 3$). No staining was observed in DRG neurones that had not been depolarized before fixation (Fig. 2*B*; $n = 3$). Staining in depolarized, non-permeabilized DRG neurones was abolished by pre-adsorption of the α_{1D} antibody with its immunizing peptide (Fig. 2*C*; $n = 3$). Incubation of the α_{1D} antibody with the α_{1C} immunizing peptide failed to inactivate the antibody and membrane staining was still observed (data not shown; $n = 3$). A lack of staining was observed in depolarized, non-permeabilized cardiac myocytes that had been incubated with preimmune serum (data not shown; $n = 3$). No staining was observed in cardiac myocytes that were not depolarized in the presence of antibody (Fig. 2*E*; $n = 3$). A schematic diagram indicating the distortion of the secondary structure of the calcium channel following depolarization to expose the antibody-binding site to the exofacial side of the membrane is shown in Fig. 2*F*.

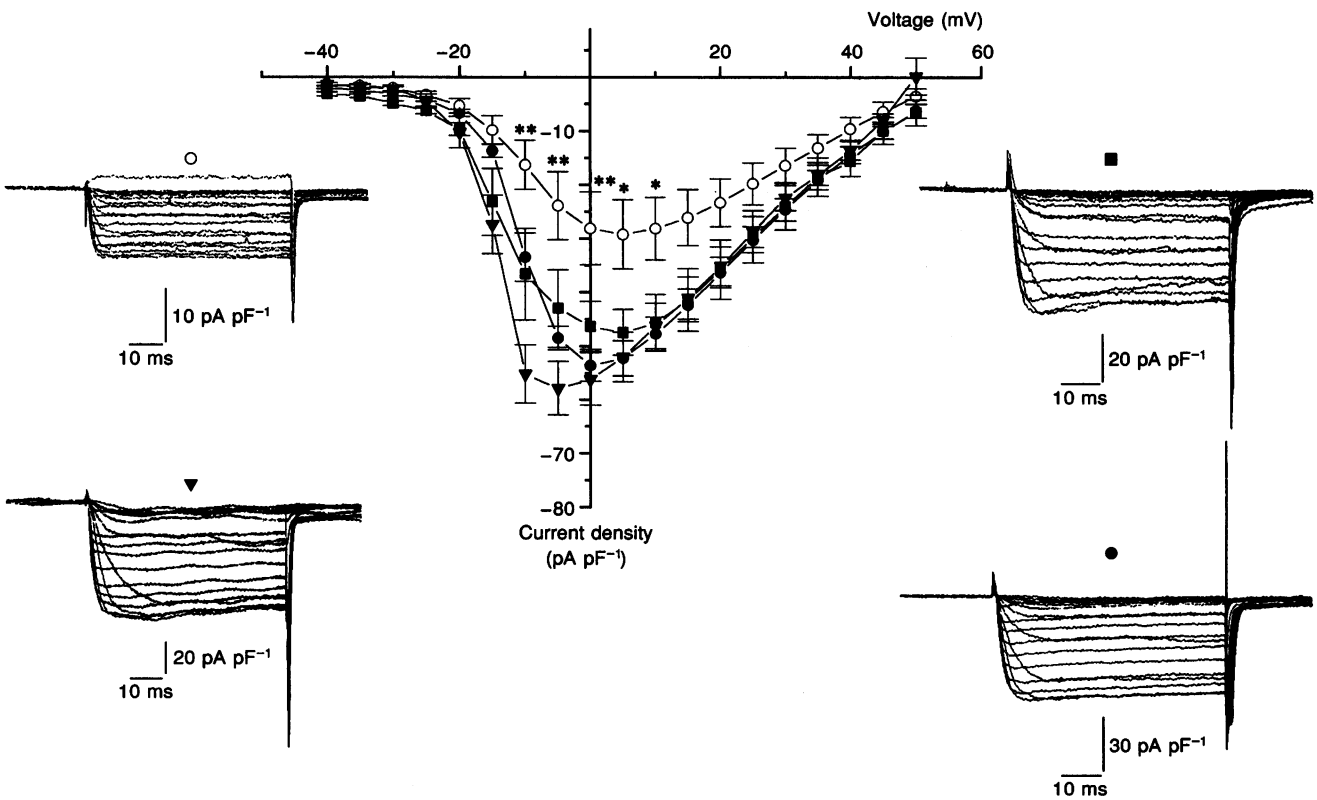

Figure 4. Current density–voltage relationships for inward I_{Ba}

Current density–voltage relationships in cultured rat DRG neurones following depolarization in the presence of α_{1D} antibody with and without immunizing peptide for 2–4 h at room temperature, and in non-depolarized neurones under the same conditions. ○, DRG neurones depolarized in the presence of the α_{1D} antibody ($n = 7$); ▼, DRG neurones that were not depolarized in the presence of the α_{1D} antibody ($n = 7$); ■, DRG neurones depolarized in the presence of the α_{1D} antibody that had been pre-adsorbed with its immunizing peptide (100 μg ml^{-1}; $n = 6$); ●, DRG neurones that were not depolarized in the presence of α_{1D} antibody pre-adsorbed with its immunizing peptide (100 μg ml^{-1}; $n = 6$). Representative example currents are shown in the insets. * $P < 0{\cdot}05$, ** $P < 0{\cdot}005$.

The specificity of the anti-α_{1D} antiserum was tested using a COS-7 cell expression system. α_{1C} was expressed and the cells were immunostained, following permeabilization, using an α_{1C}-specific antibody. Membrane-localized staining was apparent in these cells (Fig. 3*A*; $n = 7$ cells). In contrast, the α_{1D} antiserum did not stain permeabilized COS-7 cells which had been transfected with cDNA encoding α_{1C} subunits (Fig. 3*B*; $n = 27$ cells). However, α_{1D} immunostaining was observed in COS-7 cells transfected with cDNA encoding the α_{1D} subunit (Fig. 3*C*; $n = 5$ cells).

Effect of an α_{1D} antibody on calcium channel currents in DRG neurones and cardiac myocytes

In order to determine the total proportion of L-type current in cultured DRGs, the effect of nicardipine was examined in control cells. One micromolar nicardipine inhibited the maximum calcium channel current (barium current, I_{Ba}) by 59·8 ± 6·0% ($n = 6$). I_{Ba} was then examined in DRG neurones which had been incubated for 2–4 h with the α_{1D} antibody under depolarizing conditions, and compared with current in non-depolarized neurones incubated with the antibody. Further controls were performed, using antibody pre-adsorbed with its immunizing peptide. Current–voltage relationships were measured and it was observed that the I_{Ba} densities in DRG neurones that were depolarized in the presence of the α_{1D} antibody were significantly attenuated ($P < 0·005$–$0·05$) between −10 and +10 mV (Fig. 4), compared with controls. I_{Ba} at 0 mV for neurones incubated under depolarizing conditions with α_{1D} antibody was 28·5 ± 7·2 pA pF^{-1} ($n = 7$); this was 49% smaller ($P < 0·005$) than I_{Ba}, at 0 mV, of neurones incubated with the antibody in non-depolarizing media (56·2 ± 5·3 pA pF^{-1}). I_{Ba}, at 0 mV, for cells incubated with antibody that had been pre-adsorbed with its immunizing peptide in both depolarizing and non-depolarizing media was not significantly different from that of neurones incubated with antibody alone in non-depolarizing media, 46·4 ± 4·6 pA pF^{-1} ($n = 6$) and 53·6 ± 3·0 pA pF^{-1} ($n = 6$), respectively. The current waveforms from the four groups of neurones appeared to be very similar (see example currents, Fig. 4, insets); all were relatively non-inactivating over 50 ms. However, there was an approximate 10 mV difference in peak current density between neurones that were depolarized in the presence of antibody and neurones that were not depolarized in the presence of antibody. In contrast to these results, the α_{1C} antibody did not significantly reduce current density in cultured rat DRGs under depolarizing conditions: 49·7 ± 6·3 pA pF^{-1} ($n = 6$) compared with 52·5 ± 3·6 pA pF^{-1} for cells incubated under the same conditions with α_{1C} antibody pre-adsorbed with its immunizing peptide. Calcium channel currents (calcium current, I_{Ca}) were also investigated in cardiac myocytes, under the same conditions, except that preimmune α_{1D} serum was used as a control rather than α_{1D} antiserum pre-adsorbed with its immunizing peptide. A similar pattern of inhibition was observed, although to a smaller extent. The α_{1D} antibody only attenuated I_{Ca} in myocytes that had been depolarized in its presence (Fig. 5). At +10 mV, current densities were attenuated by 30% ($P < 0·05$), 5·0 ± 0·3 pA pF^{-1} compared with 7·0 ± 1·0 pA pF^{-1} in cells depolarized in the presence of preimmune serum.

Effect of α_{1D} antibody on DHP sensitivity of I_{Ba} in DRG neurones

It has been demonstrated previously that the VDCC α_{1D} subunit gives rise to an L-type current that is sensitive to

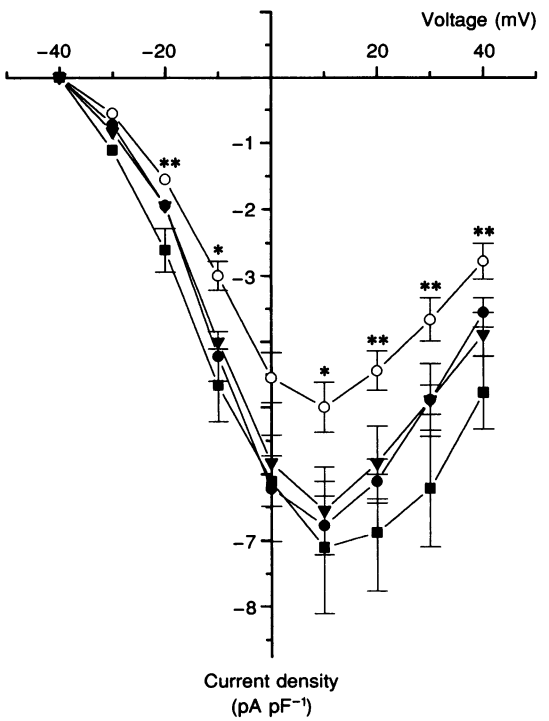

Figure 5. Current density–voltage relationships for inward I_{Ca}

Current density–voltage relationships in isolated cardiac myocytes following depolarization in the presence of α_{1D} antibody or preimmune serum for 2–4 h at room temperature, and in myocytes that were not depolarized under the same conditions. ○, cardiac myocytes depolarized in the presence of the α_{1D} antiserum ($n = 27$); ▼, cardiac myocytes that were not depolarized in the presence of the α_{1D} antiserum ($n = 16$); ●, cardiac myocytes that were not depolarized in the presence of preimmune α_{1D} serum ($n = 17$); ■, cardiac myocytes depolarized in the presence of preimmune α_{1D} serum ($n = 6$). * $P < 0·05$, ** $P < 0·005$.

modulation by DHPs (Hui *et al.* 1991). Results presented here show that an α_{1D} antibody raised against a VDCC region postulated to be involved in DHP agonist sensitivity (Grabner *et al.* 1996) inhibited I_{Ba} in cultured rat DRG neurones (Fig. 4). Therefore, we carried out experiments to investigate whether this α_{1D} antibody interfered with the DHP agonist sensitivity of I_{Ba} in these DRG neurones. *S*-(−)-Bay K 8644 (1 μM) reversibly enhanced peak I_{Ba} in DRG neurones incubated under depolarizing conditions with α_{1D} antibody that had been pre-adsorbed with its immunizing peptide by 75·0 ± 19·4% ($n = 5$; see Fig. 6*A* for an example time course). This was a significantly larger increase in current ($P < 0·05$) than that observed when 1 μM *S*-(−)-Bay K 8644 was applied to DRG neurones that had been incubated under depolarizing conditions with the α_{1D} antibody alone. Under these conditions, peak current density was reversibly enhanced by only 14·6 ± 3·5% ($n = 5$; see Fig. 6*B* for an example time course).

Immunolocalization of α_{1D} subunits following prolonged depolarization in the presence of antibody

We examined whether incubation for 2 h with the α_{1D} antibody, as was performed in the electrophysiological experiments, would induce internalization of the antibody-bound calcium channels, as this could be responsible for the reduction in current density. DRG neurones were depolarized in the presence of α_{1D} antibody and its distribution was then examined following fixation. Membrane α_{1D} immunoreactivity was still observed following prolonged (2 h) depolarization in the presence of the α_{1D} antibody both in neurones that were subsequently permeabilized (Fig. 7*A*; $n = 5$ cells) and those that were not (Fig. 7*B*, $n = 5$ cells). Experiments also showed that under non-depolarizing conditions, with α_{1D} antibody added after fixation and permeabilization, the α_{1D} subunits were largely located at the plasma membrane ($n = 3$). This suggested that depolarization for 2 h with α_{1D} antibody present caused little internalization of the α_{1D} VDCCs.

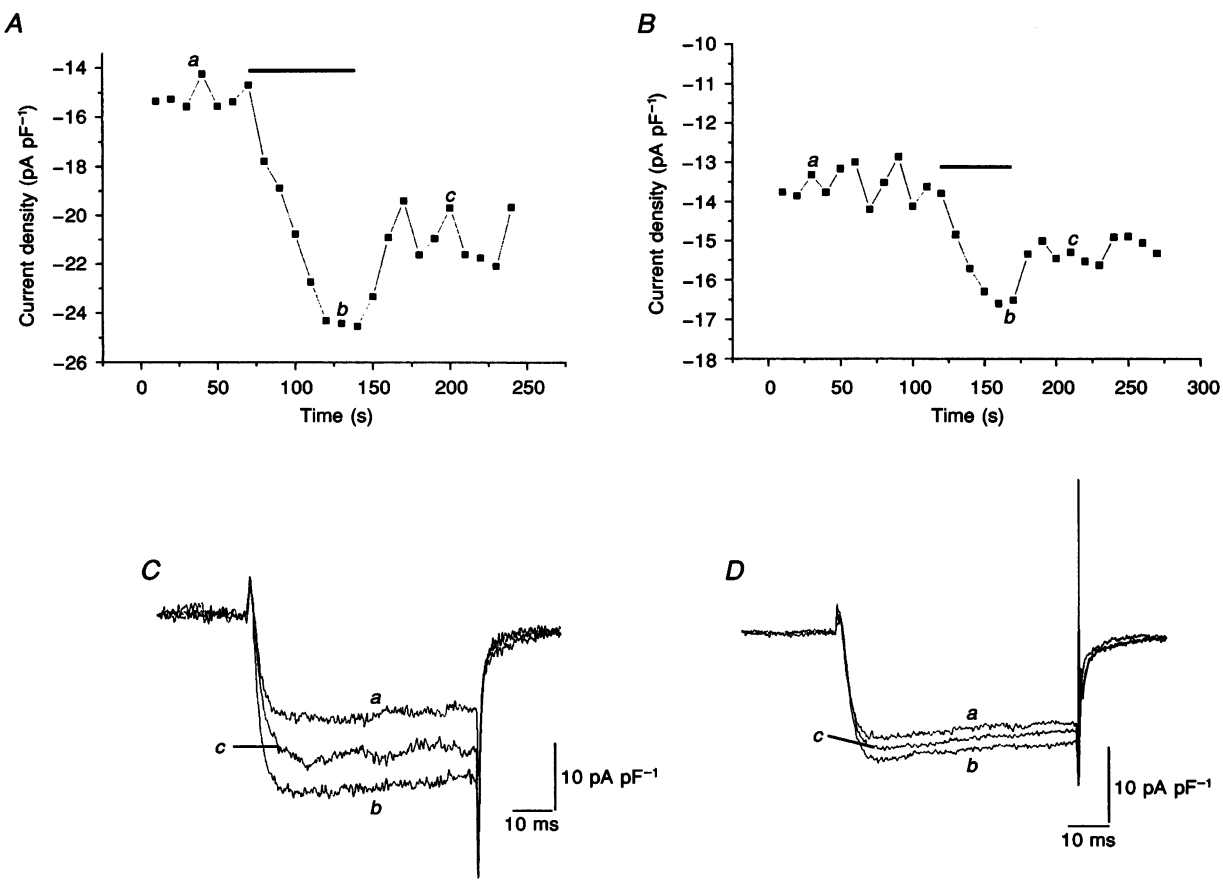

Figure 6. Effect of 1 μM *S*-(−)-Bay K 8644 on peak I_{Ba} in cultured rat DRG neurones that were depolarized in the presence of either α_{1D} antiserum alone or α_{1D} antiserum pre-adsorbed with its immunizing peptide

A, time course of peak current density in a DRG neurone depolarized in the presence of α_{1D} antiserum pre-adsorbed with its immunizing peptide (100 μg ml^{-1}). *B*, time course of peak current density in a DRG neurone depolarized in the presence of α_{1D} antiserum alone. In *A* and *B*, the horizontal bar represents the period of application of *S*-(−)-Bay K 8644. *C*, example currents corresponding to the points on the time course in *A*. *D*, example currents corresponding to the points on the time course in *B*.

DISCUSSION

As described previously, VDCC α_{1D} subunits are present in a variety of cell types in central neurones (Hell *et al.* 1993), particularly in areas responsible for neuroendocrine function, such as the hypothalamus and the pituitary gland (Chin *et al.* 1992). The results presented here demonstrate the presence of VDCC α_{1D} subunit mRNA and protein in cultured rat DRG neurones and guinea-pig cardiac myocytes using RT-PCR and immunocytochemistry. Given that the release of substance P from DRG neurones is sensitive to modulation by DHPs (Holz *et al.* 1989), it is likely that α_{1D} subunits are important in the DRG neurosecretory process. The major L-type channel in cardiac myocytes has been postulated to be α_{1C}, but the results presented in this paper clearly demonstrate the additional presence of α_{1D}.

The anti-α_{1D} antiserum used was targeted to a sequence located C-terminal to the SS1–SS2 pore-lining region between IVS5 and IVS6, in a loop predicted to be extracellular (Hui *et al.* 1991). However, immunostaining in both cell types was only observed following depolarization with 30 mM K^+. This suggested that, following depolarization, the α_{1D} subunit had undergone a conformational

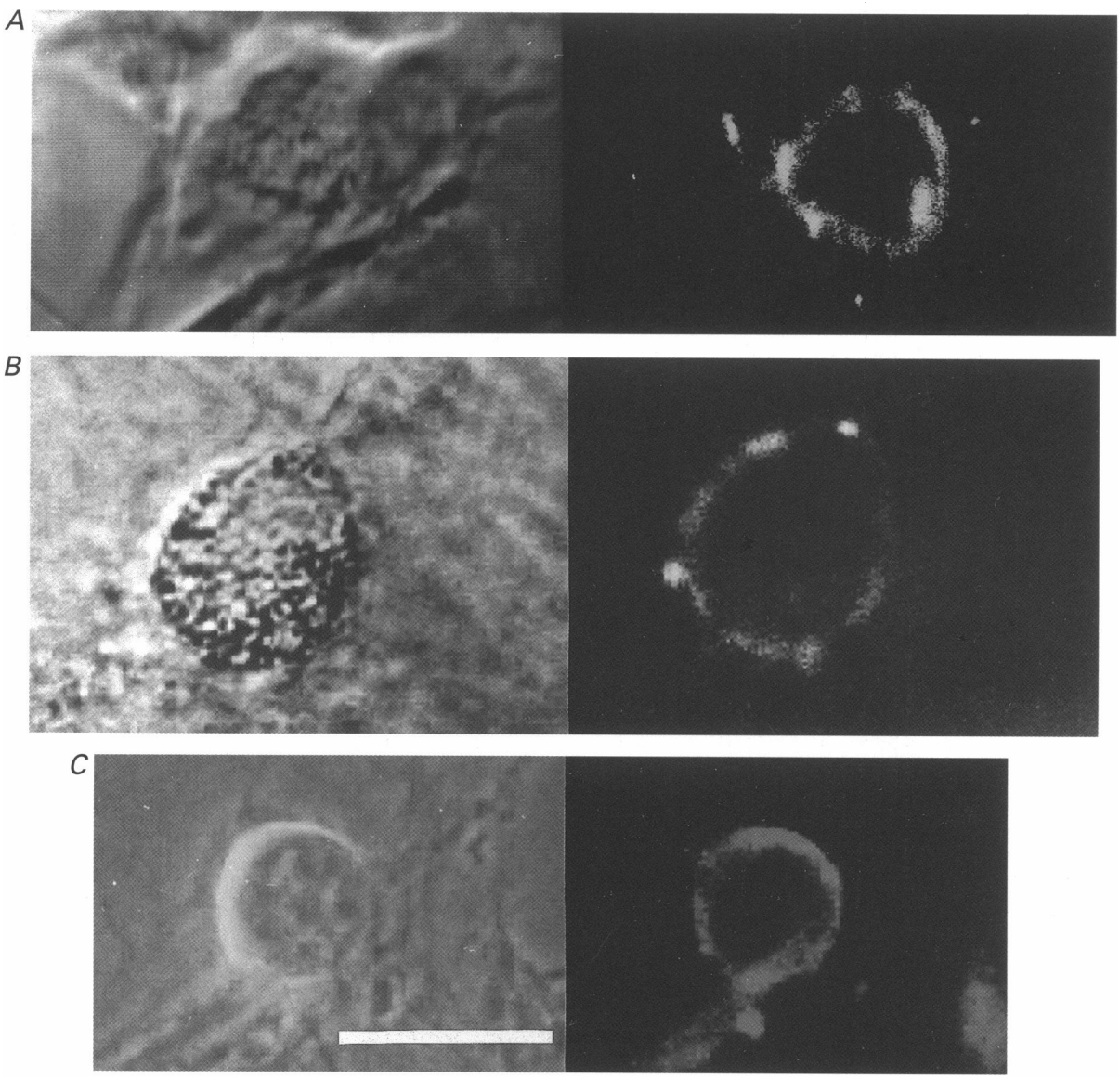

Figure 7. Confocal immunocytochemical localization of VDCC α_{1D} immunoreactivity in DRG neurones following prolonged depolarization in the presence of anti-α_{1D} antiserum

A, membrane immunolocalization of α_{1D} subunits following depolarization in the presence of the anti-α_{1D} antiserum. The DRG neurone was permeabilized. *B*, as for *A* but the cell was not permeabilized. *C*, membrane immunolocalization of α_{1D} in non-depolarized, permeabilized DRG neurones; antiserum was added after fixation. Scale bar in *C* applies to *A* and *B* and represents 15 μm. In *A–C*, the phase image is shown to the left and the fluorescence confocal image to the right.

change resulting in the relocation of the antibody-recognition site from an inaccessible location within the pore to an exofacial position where the antibody could bind. These results are similar to those observed with an α_{1A}-selective antibody targeted to a pore-flanking region in domain III (Brice *et al.* 1997). There are several potential mechanisms by which this conformational change may have occurred. It has been shown using cysteine scanning mutagenesis that two charged S4 residues in a sodium channel move from an internal position to an externally accessible location upon depolarization (Yang, George & Horn, 1996). This conformational change is described as a movement of the charged S4 membrane-spanning region perpendicular to the plane of the membrane although others have suggested other types of conformational reorientation such as an uncoiling of helical S4 regions in response to changes in membrane potential (Guy & Conti, 1990). The loops between S5 and S6 in all four transmembrane domains are believed to bend back into the interior of the channel (Varadi, Mori, Mikala & Schwartz, 1995). This region has been termed the SS1–SS2 or P loop and is thought to form the lining of the channel pore. In addition, it has been demonstrated that mutation of individual glutamate residues in the SS2 region alters the Ca^{2+} selectivity of the L-type VDCC ion conducting pathway (Tang, Mikala, Bahinski, Yatani, Varadi & Schwartz, 1993; Yang, Ellinor, Sather, Zhang & Tsien, 1993). Thus, it is feasible that depolarization and movement of the S4 regions may cause a conformational change in this pore-lining region, resulting in a consequent conformational transition of the region C-terminal to the SS2 domain, exposing the antibody-binding site. Alternatively, it has been shown that regions of transmembrane domains III and IV of an L-type VDCC α_1-subunit are implicated in the activation gating of the channel (Wang, Grabner, Berjukow, Savchenko, Glossmann & Hering, 1995). Depolarization may lead to conformational changes here, which in turn may promote a change which exposes the antibody-recognition site. Indeed, a conformational change in this VDCC region is likely to underlie the voltage-dependent binding and actions of DHP antagonists (Hamilton, Yatani, Brush, Schwartz & Brown, 1987).

The electrophysiological data presented here demonstrate that when the anti-α_{1D} antibody bound to its target calcium channel, I_{Ba} was significantly attenuated. The mechanism by which this occurs is not clear. However, there are several possibilities. (1) The antibody binds close to the α_{1D} pore region. Therefore, it is conceivable that it may in some way occlude the 'mouth' of the pore and inhibit current from passing through channels formed from α_{1D} subunits. (2) The antibody only binds to channels following depolarization. It may render them incapable of passing current by 'locking' them in an inactivated state. (3) The binding of the antibody to the α_{1D} subunit may cause the channel to be internalized, thereby reducing current density. This last possibility is considered to be unlikely, however, as membrane staining of α_{1D} antibody was still observed after DRG neurones had been incubated with α_{1D} antibody under depolarizing conditions for 2 h. Thus α_{1D} subunits were still present in the membrane after this period. Receptor internalization is thought to play a significant role in the mechanism of action of Lambert Eaton Myasthenic Syndrome (LEMS) antisera, some of which are directed against calcium channel epitopes. However, the majority of these studies required overnight incubation of tissue with the antisera for inhibition of calcium channel current to be seen (Grassi, Magnelli, Carabelli, Sher & Carbone, 1994). It has been postulated that LEMS antisera cause a reduction in calcium channel currents by inducing channel internalization (see Peers, Lang, Newsom-Davis & Wray, 1990), although, more recently, Garcia, Mynlieff, Sanders, Walrond & Beam (1996) have concluded that some of the effect may be due to a reduction in single channel conductance. Indeed, some workers have observed inhibition after much shorter times of incubation (Kim & Neher, 1988) and this may be as a result of a direct inhibitory effect on the channels. Our evidence suggests that incubation of cultured DRGs with α_{1D} antibody for 2 h is not a sufficient period of time to induce calcium channel internalization.

In addition to being located close to a region which contributes to the α_{1D} VDCC pore, the region to which the antibody binds is in a domain that was found to confer DHP agonist sensitivity to DHP-insensitive calcium channels (Grabner *et al.* 1996). Consequently, it was unsurprising to find that, in addition to attenuating whole-cell VDCC current, the anti-α_{1D} antibody reduced the ability of the DHP agonist *S*-(−)-Bay K 8644 to potentiate I_{Ba} in DRG neurones. This could have been caused either by the antibody directly interfering with the binding of the DHP agonist or perhaps by interfering with the allosteric interaction between a Ca^{2+}-binding site in the pore and a DHP-binding domain (Mitterdorfer, Sinnegger, Grabner, Streissnig & Glossmann, 1995). Alternatively, it may be that the antibody has rendered the α_{1D} channels non-conducting and that no current can pass through them at all, thus *S*-(−)-Bay K 8644 could have no effect on these currents. In DRG neurones we have shown that the proportion of L-type current, determined by the extent of inhibition of the maximum I_{Ba} by 1 μM nicardipine, is about 60%. In the present experiments we have demonstrated that exposure to a selective α_{1D} antibody reduces current by about 50%, indicating that the residual 10% L-type current is not associated with α_{1D} and is probably carried by α_{1C} channels.

In conclusion, we have demonstrated the presence of an α_{1D} subunit in cultured rat DRG neurones and guinea-pig cardiac myocytes. Furthermore, amino acids 1417–1434 of the α_{1D} subunit are not exposed on the extracellular face of these cells unless the cell membranes are depolarized. An antibody targeted to these amino acids inhibited whole-cell VDCC currents in both cell types and also interfered with the actions of the DHP agonist *S*-(−)-Bay K 8644 in DRG neurones.

AKHA, A. A. S., WILLMOTT, N. J., BRICKLEY, K., DOLPHIN, A. C., GALIONE, A. & HUNT, S. V. (1996). Anti-Ig-induced calcium influx in rat B lymphocytes mediated by cGMP through a dihydropyridine-sensitive channel. *Journal of Biological Chemistry* **271**, 7297–7300.

BIRNBAUMER, L., CAMPBELL, K. P., CATTERALL, W. A., HARPOLD, M. M., HOFMANN, F., HORNE, W. A., MORI, Y., SCHWARTZ, A., SNUTCH, T. P., TANABE, T. & TSIEN, R. W. (1994). The naming of voltage-gated calcium channels. *Neuron* **13**, 505–506.

BRICE, N. L., BERROW, N. S., CAMPBELL, V., PAGE, K. M., BRICKLEY, K., TEDDER, I. & DOLPHIN, A. C. (1997). Importance of the different β subunits in the membrane expression of the α1A and α2 calcium channel subunits: studies using a depolarisation sensitive antibody. *European Journal of Neuroscience* **9**, 749–759.

BRICKLEY, K., CAMPBELL, V., BERROW, N., LEACH, R., NORMAN, R. I., WRAY, D., DOLPHIN, A. C. & BALDWIN, S. A. (1995). Use of site directed antibodies to probe the topography of the alpha 2 subunit of voltage-gated Ca^{2+} channels. *FEBS Letters* **364**, 129–133.

CAMPBELL, V., BERROW, N. S., BRICKLEY, K., PAGE, K., WADE, R. & DOLPHIN, A. C. (1995*a*). Voltage-dependent calcium channel β-subunits in combination with α_1 subunits have a GTPase activating effect to promote hydrolysis of GTP by $G_{\alpha o}$ in rat frontal cortex. *FEBS Letters* **370**, 135–140.

CAMPBELL, V., TERRACCIANO, C. M. N., NAQVI, R. U., BRICKLEY, K., BALDWIN, S. A., MACLEOD, K. T. & DOLPHIN, A. C. (1995*b*). An antibody to L-type calcium channels reduces voltage-dependent calcium current in guinea-pig cardiac myocytes in a voltage-dependent manner. *Journal of Physiology* **487.P**, 130*P*.

CATTERALL, W. A. & STREISSNIG, J. (1992). Receptor sites for calcium antagonists. *Trends in Pharmacological Sciences* **13**, 252–262.

CHIN, H., SMITH, M. A., KIM, H. L. & KIM, H. (1992). Expression of dihydropyridine-sensitive brain calcium channels in the rat central nervous system. *FEBS Letters* **299**, 69–74.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

DOLPHIN, A. C. (1995). Voltage-dependent calcium channels and their modulation by neurotransmitters and G proteins. *Experimental Physiology* **80**, 1–36.

GARCIA, K. D., MYNLIEFF, M., SANDERS, D. B., WALROND, J. W. & BEAM, K. G. (1996). Lambert-Eaton sera reduce low-voltage and high-voltage activated calcium currents in murine dorsal root ganglion neurons. *Proceedings of the National Academy of Sciences of the USA* **93**, 9264–9269.

GRABNER, M., WANG, Z., HERING, S., STREISSNIG, J. & GLOSSMANN, H. (1996). Transfer of 1,4-dihydropyridine sensitivity from L-type to class A (BI) calcium channels. *Neuron* **16**, 207–218.

GRASSI, C., MAGNELLI, V., CARABELLI, V., SHER, E. & CARBONE, E. (1994). Inhibition of low and high-threshold Ca^{2+} channels of human neuroblastoma IMR32 cells by Lambert-Eaton myasthenic syndrome (LEMS) IgGs. *Neuroscience Letters* **181**, 50–56.

GUY, H. R. & CONTI, F. (1990). Pursuing the structure and function of voltage-gated channels. *Trends in Neurosciences* **13**, 201–206.

HAMILTON, S. L., YATANI, A., BRUSH, K., SCHWARTZ, A. & BROWN, A. M. (1987). A comparison between the binding and the electrophysiological effects of dihydropyridines on cardiac membranes. *Molecular Pharmacology* **31**, 221–231.

HELL, J. W., WESTENBROEK, R. E., WARNER, C., AHLIJANIAN, M. K., PRYSTAY, W., GILBERT, M. M., SNUTCH, T. P. & CATTERALL, W. A. (1993). Identification and subcellular localisation of the neuronal class C and class D L-type calcium channel α subunits. *Journal of Cell Biology* **123**, 949–962.

HOLZ, G. G., KREAM, R. M., SPEIGEL, A. & DUNLAP, K. (1989). G proteins couple α-adrenergic and $GABA_B$ receptors to inhibition of peptide secretion from peripheral sensory neurons. *Neuroscience* **9**, 657–666.

HUI, A., ELLINOR, P. T., KRIZANOVA, O., WANG, J.-J., DIEBOLD, R. J. & SCHWARTZ, A. (1991). Molecular cloning of multiple subtypes of a novel brain isoform of the α_1 subunit of the voltage-dependent calcium channel. *Neuron* **7**, 35–44.

JANIS, R. A. & TRIGGLE, D. J. (1991). Drugs acting on calcium channels. In *Calcium Channels: Their Properties, Functions, Regulation and Clinical Relevance*, ed. HURWITZ, L. H., PARTRIDGE, L. D. & LEACH, J. K., pp. 195–249. CRC Press, Boca Raton, USA.

KASS, R. S., ARENA, J. P. & CHIN, S. (1991). Block of L-type calcium channels by charged dihydropyridines. *Journal of General Physiology* **98**, 63–75.

KIM, H. L., KIM, H., LEE, P., KING, R. G. & CHIN, H. (1992). Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel α2 subunit. *Proceedings of the National Academy of Sciences of the USA* **89**, 3251–3255.

KIM, Y. I. & NEHER, E. (1988). IgG from patients with Lambert-Eaton syndrome blocks voltage-dependent calcium channels. *Science* **239**, 405–408.

MIKAMI, A., IMOTO, K., TANABE, T., NIDOME, T., MOTI, Y., TAKESHIMA, H., NARUMIYA, S. & NUMA, S. (1989). Primary structure and functional expression of the cardiac dihydropyridine sensitive calcium channel. *Nature* **340**, 230–233.

MITTERDORFER, J., SINNEGGER, M. J., GRABNER, M., STREISSNIG, J. & GLOSSMANN, H. (1995). Coordination of Ca^{2+} by the pore region glutamates is essential for high affinity dihydropyridine binding to the cardiac Ca^{2+} channel α_1 subunit. *Biochemistry* **34**, 9350–9355.

MORI, Y., FREIDRICH, T., KIM, M.-S., MIKAMI, A., NAKAI, J., RUTH, P., BOSSE, E., HOFMANN, F., FLOCKERZI, V., FURUICHI, T., MIKOSHIBA, K., IMOTO, K., TANABE, T. & NUMA, S. (1991). Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* **350**, 398–402.

NAKAYAMA, H., TAKI, M., STREISSNIG, J., GLOSSMANN, H., CATTERALL, W. A. & KANAOKA, Y. (1991). Identification of 1,4-dihydropyridine binding regions within the α_1 subunit of skeletal muscle calcium channels by photoaffinity labelling. *Proceedings of the National Academy of Sciences of the USA* **88**, 9203–9207.

PEERS, C., LANG, B., NEWSOM-DAVIS, J. & WRAY, D. W. (1990). Selective action of myasthenic syndrome antibodies on calcium channels in a rodent neuroblastoma × glioma cell line. *Journal of Physiology* **421**, 293–308.

PRAGNELL, M., SAKAMOTO, J., JAY, S. D. & CAMPBELL, K. P. (1991). Cloning and tissue specific expression of the brain calcium channel β subunit. *FEBS Letters* **291**, 253–258.

REGULLA, S., SCHNEIDER, T., NASTAINCZYCK, W., MEYER, H. E. & HOFMANN, F. (1991). Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and azidopine with the calcium channel α_1 subunit. *EMBO Journal* **10**, 45–49.

SCHUSTER, A., LACINOVA, L., KLUGBAUER, N., ITO, H., BIRNBAUMER, L. & HOFMANN, F. (1996). The IVS6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenalkylamines. *EMBO Journal* **15**, 2365–2370.

SNUTCH, T. P., TOMLINSON, W. J., LEONARD, J. P. & GILBERT, M. M. (1991). Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. *Neuron* **7**, 45–57.

STREISSNIG, J., MURPHY, B. J. & CATTERALL, W. A. (1991). Dihydropyridine receptor of L-type Ca^{2+} channels: Identification of binding domains for [^{3}H](+)-PN200-110 and [^{3}H]azidopine within the α_1 subunit. *Proceedings of the National Academy of Sciences of the USA* **88**, 10769–10773.

STRUBING, C., HERING, S. & GLOSSMANN, H. (1993). Evidence for an external location of the dihydropypridine agonist receptor site on smooth muscle and skeletal muscle calcium channels. *British Journal of Pharmacology* **108**, 884–891.

TANG, S., MIKALA, G., BAHINSKI, A., YATANI, A., VARADI, G. & SCHWARTZ, A. (1993). Molecular localization of ion selectivity sites within the pore of a human L-type cardiac calcium channel. *Journal of Biological Chemistry* **268**, 13026–13029.

TANG, S., YATANI, A., BAHINSKI, A., MORI, Y. & SCHWARTZ, A. (1993). Molecular localisation of regions in the L-type calcium channel critical for dihydropyridine action. *Neuron* **11**, 1013–1021.

VARADI, G., MORI, Y., MIKALA, G. & SCHWARTZ, A. (1995). Molecular determinants of Ca^{2+} channel function and drug action. *Trends in Pharmacological Sciences* **16**, 43–49.

WANG, Z., GRABNER, M., BERJUKOW, S., SAVCHENKO, A., GLOSSMANN, H. & HERING, S. (1995). Chimeric L-type calcium channels expressed in *Xenopus laevis* oocytes reveal role of repeats III and IV in activation gating. *Journal of Physiology* **486**, 131–137.

WYATT, C. N., BRICKLEY, K. & DOLPHIN, A. C. (1996). An antibody to an L-type calcium channel attenuates voltage-dependent barium currents in cultured rat dorsal root ganglion cells. *Journal of Physiology* **495.P**, 79*P*.

YANG, J., ELLINOR, P. T., SATHER, W. A, ZHANG, J.-F. & TSIEN, R. W. (1993). Molecular determinants of Ca^{2+} selectivity and ion permeation in L-type calcium channels. *Nature* **366**, 158–161.

YANG, N., GEORGE, A. L. JR & HORN, R. (1996). Molecular basis of charge movement in voltage-gated sodium currents. *Neuron* **16**, 113–122.

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