Use of site-directed antibodies to probe the topography of the α_2 subunit of voltage-gated Ca^{2+} channels

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Received 6 February 1995; revised version received 27 March 1995

Abstract Polyclonal antibodies were raised against peptides corresponding to residues 1–15, 469–483 and 933–951 of the rabbit skeletal muscle L-type calcium channel α_2/δ primary translation product, for use as topological probes. Immunocytochemical comparison of the abilities of the antibodies to bind to the α_2 and δ subunits in intact and detergent-permeabilised rat dorsal root ganglion cells enabled the membrane orientation of these regions to be established. The resultant data indicate that the regions containing residues 1–15 and 469–483 of the α_2 subunit, and residues 1–17 of the δ subunit, are exposed on the extracellular surface of the membrane, findings consistent with a model that proposes α_2 to be entirely extracellular.

Key words: Calcium channel; α_2 subunit; t-Tubule; Dorsal root ganglion

1. Introduction

The L-type, dihydropyridine (DHP)-sensitive, voltage-gated calcium channels of mammalian skeletal muscle consist of five distinct polypeptides, α_1, α_2, β, γ and δ, the sequence of each of which has been established by cDNA cloning [1,2]. Each subunit is encoded by a separate gene, with the exception of the α_2/δ primary transcript, the δ subunit arising as a result of proteolysis from the C-terminal end of the α_2/δ polypeptide [3]. The α_1 subunit contains the ion channel itself, and the binding sites for ligands such as 1,4-dihydropyridines, while the β subunit appears to be necessary for expression of the native kinetic characteristics of the channel [4–6] and to be involved in channel regulation by phosphorylation events [7]. The roles of the other subunits are less clear, although co-expression of the α_2/δ subunit enhances the calcium channel activity induced by injection of *Xenopus* oocytes with cardiac L-type α_1 subunit mRNA [8]. The α_2 polypeptide possibly exerts this effect by helping to target the α_1 subunit to the plasma membrane or by stabilising it in that location. Similar findings have also been made for α_{1A} [9] and α_{1B} [10] calcium channels, suggesting that the α_2 subunit plays an important role in the function of several types of voltage-gated calcium channel. mRNA transcripts that hybridise with α_2 cDNA probes are detectable in many tissues, including cardiac muscle, aorta, ileal smooth muscle, lung and brain [11,12]. Unlike calcium channel α_1 subunits, for which several different genes have now been identified, the slightly different α_2 isoforms expressed in brain and skeletal muscle appear to be splice variants of a single α_2/δ gene [12].

Despite the apparent functional importance of α_2/δ, little is known about its secondary and tertiary structure. However, its cloning in 1988 allowed the proposal of a model for its putative arrangement in the membrane, based upon hydropathic analysis of the deduced amino acid sequence [11]. After taking into account the subsequent discovery that both the α_2 and δ subunits are encoded within the same primary transcript, this model predicts that the α_2 subunit contains two membrane-spanning segments (Fig. 1A). The large, hydrophilic N-terminal domain and a much shorter C-terminal domain are predicted to be extracellular, while a large hydrophilic domain lying between these two regions is predicted to be located on the cytoplasmic side of the membrane. In contrast, a more recent model predicts that the entire α_2 subunit is extracellular and is bound to the membrane via disulphide bonds to the δ subunit, which has a single putative membrane-spanning region, an exofacial N-terminus and an endofacial C-terminus (Fig. 1B) [13]. This model is based upon the observation that the α_2 subunit is both highly glycosylated [14,15] and can be released from microsomal membranes at high pH under reducing but not under non-reducing conditions [13].

In order to provide direct evidence for the arrangement of the protein in the membrane, and thus allow discrimination between the two models, we have raised polyclonal antibodies against the regions of rabbit skeletal muscle α_2/δ sequence [11] corresponding to residues 1–15, 469–483 and 933–951. The sequence 1–15 is conserved at all but one, and the sequence 933–951 is conserved at all but three residues in the rabbit, human and rat subunits, while peptide 469–483 is fully conserved in all three species [11,12,16]. None of these peptides are affected by alternative splicing of the primary transcript. The latter sequence represents a potential cAMP-dependent phosphorylation site, and is located in the large intracellular loop of the model of Ellis et al. [11], while being exofacial in the model of Jay et al. [13]. We have used the antibodies as membrane impermeant probes to study the orientation of these regions of both the α_2 and δ subunits in intact and detergent-permeabilised, cultured rat dorsal root ganglion (DRG) neurons. These cells contain several types of calcium channel, including L-, T-and N-type [17], and are ideally suited for immunocytochemical studies.

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SSDI 0014-5793(95)00371-1

2. Materials and methods

2.1. Preparation and characterization of antibodies

Peptides having the sequences EPFPSAVTIKSWVDKC, corresponding to α_2(1–15), with an additional C-terminal cysteinyl residue, SLEDIKRLTPRFTLC, corresponding to α_2(469–483) and EAADMEDDDFTASMSKQSC, corresponding to α_2/δ(933–951), were synthesised using the N^{α}-fluorenylmethoxycarbonyl-polyamide solid-phase method [18]. Examination of each synthetic product by either matrix-assisted Fast Atom Bombardment or Laser Desorption Mass Spectrometry revealed species having the expected molecular masses of 1,805, 1,792 and 2,079 amu, respectively. Further characterisation of the peptides by amino acid analysis and reverse-phase HPLC, using previously described methods [19], revealed that they were (90, 85 and 75)% pure, respectively, and so they were used without further purification. The peptides were coupled via their cysteine residues to ovalbumin using maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (Pierce Chemical Co. USA) and antiserum raised in female half-lop rabbits essentially as previously described [19,20]. Peptide-specific antibodies were purified by passage of the antiserum through a column of immunising peptide (2 mg peptide/ml gel) immobilised on Sulfolink coupling gel (Pierce Chemical Co. USA), followed by elution in either 0.2 M glycine/HCl, pH 2.4, or 5 M $MgCl_2$ and dialysis into 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS). The typical yields were 0.4, 1.0 and 0.2 mg/ml antiserum, respectively.

The ability of both the antisera and the affinity-purified antibodies to recognise the synthetic peptides was assessed by enzyme-linked immunosorbent assays (ELISA), performed essentially as previously described [19]. When ELISA was used to assess the ability of the antibodies to recognise the intact, native channel polypeptide, microtitre plates were coated by incubation overnight at 4°C with a solution of purified rabbit skeletal muscle DHP-receptor (140 ng/well) in PBS containing 0.0025% (w/v) digitonin. Subsequent incubation with primary antibody was for only 4 h, but otherwise ELISA was performed as for the synthetic peptide assays. For immunoblotting, proteins were separated on 4–12% gradient minigels and transferred onto ProBlott membranes (Applied Biosystems Inc.) by semi-dry blotting. Incubation with antibodies (at the dilution specified in the figure legend) and subsequent visualization were as previously described [21].

2.2. Membrane and channel preparations

T-tubule membranes were prepared from rat skeletal muscle by the method of Rosemblatt et al. [2], all steps of the procedure being carried out at 0–4°C. The L-type channel was purified from rabbit skeletal muscle according to the method of Flockerzi et al. [23].

2.3. Immunocytochemistry

Cryostat sections (12–16 μm thick) of frozen, unfixed rat biceps muscle were prepared by standard techniques and collected onto gelatin-coated glass slides. For immunostaining, the sections were incubated with either control rabbit IgG or test antibodies (20 μg/ml) for 3.5 h at 20°C or with anti-serum and preimmune serum (diluted 1/1,000) for 20 h at 4°C in PBS. After washing (5 × 3 min) with PBS, they were incubated for 1 h at 20°C with biotinylated donkey anti-rabbit IgG (Amersham) at a dilution of 1/40 in PBS. They were then washed again (5 × 3 min) and incubated with streptavidin–Texas red (1/40) in PBS for 20 min at 20°C before washing (5 × 3 min) and mounting in antifade mountant (Citifluor, City University, London). DRG neurons were isolated and cultured for 10–15 days as described previously [24]. Before immunostaining, cells were washed with 40 mM Tris-HCl, 154 mM NaCl, pH 7.4 (TBS) and then fixed by incubation in 4% paraformaldehyde in TBS for 30 min at room temperature. For permeabilisation, cells were treated for 3 × 5 min at room temperature with 0.02% Triton X-100 in TBS. Permeabilised and intact cells were then washed (3 × 5 min) in TBS containing 20% goat serum, 4% bovine serum albumin and 0.1% DL-lysine, before incubation overnight at 4°C with primary antibodies diluted as follows (anti-α_2(1–15), anti-α_2(469–483) and anti-α_2/δ(933–951) antiserum, (1/1,000); anti-β subunit antiserum [6], (1/1,500); anti-Gα_o affinity-purified antibody [24], (4 μg/ml)). For control experiments, the diluted antiserum was incubated at 37°C for 1 h with the synthetic peptide (20 μg/ml) before use. After incubation with the primary antibody, the cells were washed again (4 × 5 min) and then incubated with biotinylated goat anti-rabbit IgG (diluted 1/200) for 2 h at 4°C. Following washing (4 × 5 min) DRGs were incubated with Extravidin fluorescein isothiocyanate conjugate (diluted 1/50) for 1 h at room temperature. They were then washed (5 × 5 min) with TBS before mounting as for the muscle sections. Bound antibody was subsequently detected in both cells and muscle sections by confocal fluorescence microscopy, using a Bio-Rad MRC-600 laser scanning microscope. Optical sections of skeletal muscle were taken at 2 μm intervals in the vertical plane of the sample. Immunofluorescent intensity in DRG neurons was quantified for each antiserum, under permeabilised and nonpermeabilised conditions, as previously described [6].

All chemicals were purchased from Sigma unless otherwise stated.

3. Results and discussion

The antisera raised against synthetic peptides α_2(1–15), α_2(469–483) and α_2/δ(933–951) each showed a high titre of anti-peptide antibodies when assayed by ELISA using microtitre plate-bound immunising peptide as antigen (data not shown). It was not possible to determine whether the antisera also recognised the intact, denatured neuronal α_2 or α_2/δ polypeptides because the low abundance of these proteins in rat brain membranes and the low yield of DRGs precluded their detection by Western blotting. However, the antibodies were shown to recognise the more abundant, alternatively-spliced product found in skeletal muscle. Anti-α_2(469–483) antiserum strongly stained a single polypeptide band of apparent M_r 143,000, identical to that of the α_2 subunit, on Western blots both of rat skeletal muscle t-tubule membranes and of purified rabbit L-type channel preparations (Fig. 2 lanes a and b, respectively). No staining of this band was seen in either preparation with pre-immune serum, even when a 5-fold higher concentration of serum was used (Fig. 2, lanes c and d, respectively). The anti-α_2(469–483) antibodies purified by chromatography on a peptide affinity column showed a pattern of staining identical to that given by the antiserum, confirming their specificity for the α_2 subunit (data not shown). A similar result was obtained using purified anti-α_2(1–15) antibody (results not shown). However, antiserum against α_2/δ(933–951) failed to recognize the α_2/δ complex in immunoblots following SDS/PAGE even under non-reducing conditions (results not shown).

Although both anti-α_2(1–15) and anti-α_2(469–483) antibodies had been shown specifically to recognise the denatured channel subunit, an ability to recognise the native protein in its membrane environment is a prerequisite for use of antibodies in topological studies. This ability was investigated both by ELISA and by immunohistochemical methods. In ELISA using the purified, native channel from rabbit skeletal muscle anti-α_2(1–15) (Fig. 3 (▼)), anti-α_2(469–483) (Fig. 3 (●)) and anti-α_2/δ(933–951) (Fig. 3 (◆)) sera each reacted with plate-bound channels much more strongly than their respective pre-immune sera. Data for the most reactive of these preimmune sera are shown in Fig. 3 (○). In the same assay, plate-bound channels were also recognised more strongly by affinity-purified anti-α_2(469–483) antibodies (Fig. 3 (■)) than by control IgG (Fig. 3 (□)). Longitudinal sections of rat skeletal muscle exhibited strong staining upon incubation with affinity-purified antibodies against α_2(469–483) (Fig. 4A) followed by biotinylated anti-rabbit IgG and then streptavidin–Texas red. No staining was seen when control rabbit IgG was used (Fig. 4B). Additionally, affinity-purified antibodies against α_2(1–15) and antiserum against α_2/δ(933–951) produced similar staining in muscle sections to that shown in Fig. 4A, while the staining obtained with preimmune serum resembled that yielded by control IgG (as

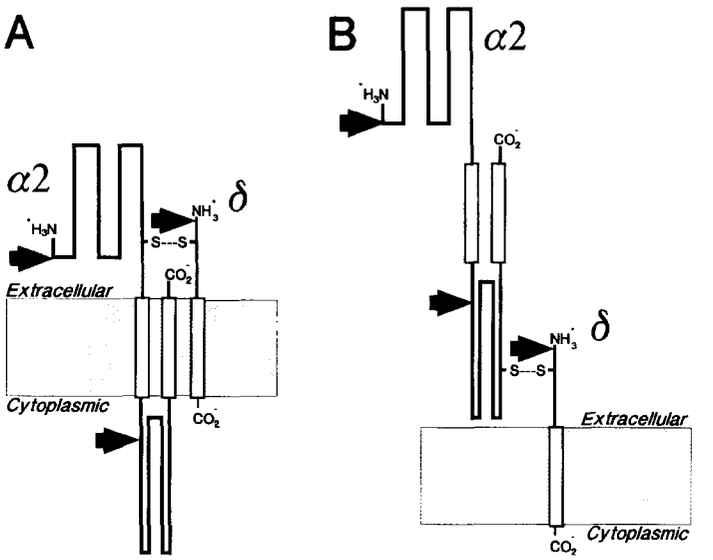

Fig. 1. Alternative models for the arrangement of the Ca^{2+} channel α_2/δ subunit complex in the t-tubule membrane. The locations of hydrophobic segments identified as potential membrane-spanning helices in the model of Ellis and co-workers [11] are indicated. The latter model takes into account the fact that the α_2 and δ subunits are encoded by the same gene [3], and is shown in (A). The model of Jay and co-workers [12] is illustrated in (B). The arrows indicate the approximate sequence locations of the synthetic peptides α_2(1–15), α_2(469–483) and α_2/δ(933–951).

Fig. 4B, results not shown). The staining pattern yielded by the antibodies consisted of striations regularly spaced at 1.8 μm intervals, running perpendicular to the long axis of the muscle fibre. Such periodicity is characteristic of the arrangement of the triad junctions in skeletal muscle, where the L-type calcium channels are located, and is identical to that reported by other workers using monoclonal antibodies against the α_1, γ and α_2 channel subunits [25,26]. The intense staining of unfixed cryosections in this manner strongly suggested that the antibodies raised against α_2(1–15), α_2(469–483) and α_2/δ(933–951) were all able to recognise the native protein. This suggestion was confirmed for all three antibodies by the ELISA experiments.

The results of the experiments on the skeletal muscle Ca^{2+} channel described above indicated that antibodies against α_2(1–15), α_2(469–483) and α_2/δ(933–951) were likely to be useful as probes of the α_2 and δ polypeptide arrangements in the membrane. However, despite the dense expression of calcium channels in the t-tubules of skeletal muscle, it was technically too difficult to obtain intact, isolated myofibres suitable for topological experiments from skeletal muscle. Instead, cultured rat DRGs, which are known to contain L-type calcium channels [17] and are more experimentally amenable were used. Although the low numbers of DRGs available precluded α_2 subunit detection by Western blotting, Triton X-100-permeabilised cells exhibited strong membrane-associated staining by the α_2(469–483) antibodies in immunofluorescence experiments (Fig. 4C). No staining was seen when pre-immune serum was employed (result not shown). The specificity of the staining was confirmed by the absence of fluorescence seen when the anti-α_2(469–483) serum was pre-treated with an excess of the synthetic peptide (result not shown). Cells which had not been treated with Triton X-100 also revealed immunostaining of the plasma membrane, following incubation with this antiserum (Fig. 4D). The pattern of staining by this antibody in permeabilised cells closely resembled that given by an antibody directed against the α-subunit of the G-protein G_o (Fig. 4E), although this antibody failed to produce any staining in cells which had not been treated with Triton X-100 (Fig. 4F). Following incubation with antibodies against the β-subunit of the calcium channel, detergent-permeabilised cells also revealed membrane staining similar to that shown in Fig. 4C (see Fig. 4G), which was not apparent in unpermeabilised cells (Fig. 4H). Since both

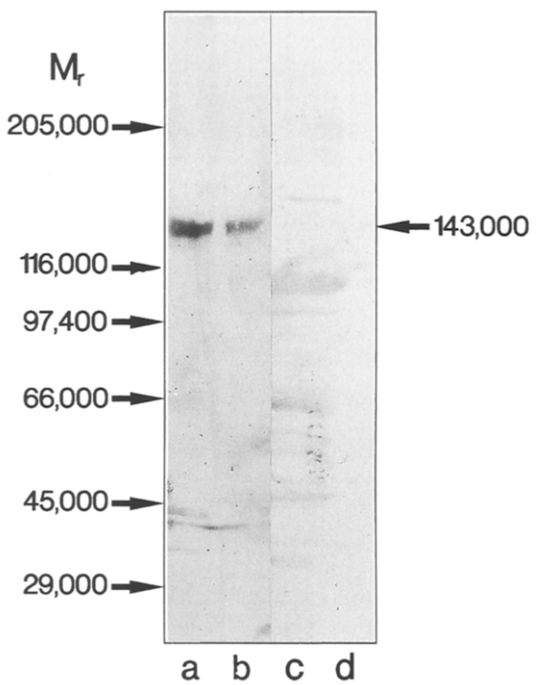

Fig. 2. Immunoblots of rat skeletal muscle t-tubule membrane proteins (50 μg) (lanes a and c) and purified rabbit L-type calcium channel proteins (1 μg) (lanes b and d) stained either with antiserum against peptide α_2(469–483) (1 in 500 dilution, lanes a and b) or with pre-immune serum (1 in 100 dilution, lanes c and d). Arrows indicate the positions of molecular weight markers.

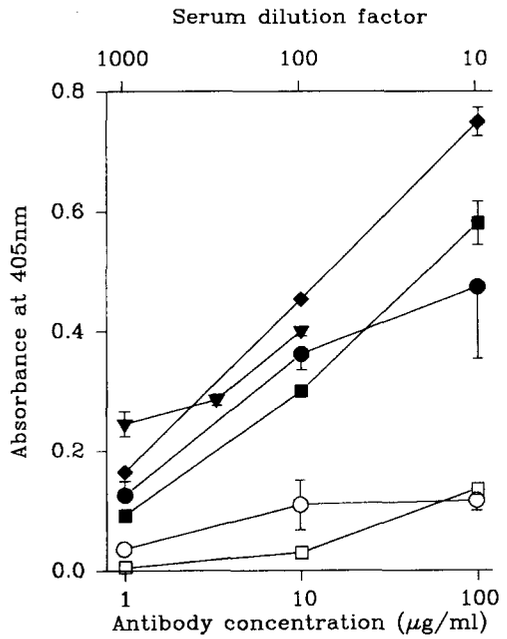

Fig. 3. Recognition of the intact rabbit skeletal muscle L-type calcium channel in ELISA by the anti-α_2/δ antisera and affinity purified antibodies. Plates coated with the purified channel protein were incubated with serial dilutions of either pre-immune serum (○) or serum raised against α_2(1–15) (▼), α_2(469–483) (●) or α_2/δ(933–951) (◆), or with various concentrations of affinity-purified anti-α_2(469–483) antibodies (■) or control rabbit IgG (□). Each point is the mean absorbance at 405 nm of triplicate samples, ± S.D.

of these proteins are known to be located at the cytoplasmic surface of the plasma membrane [21,27], the lack of staining produced by these antibodies in cells which had not been treated with Triton X-100, confirmed the integrity of the plasma membrane barrier in the fixed cells. Intact cells also showed membrane-associated staining following incubation with anti-α_2(1–15) (Fig. 4I) and anti-α_2/δ(933–951) serum (Fig. 4J), which was removed following preincubation with the immunising peptide (results not shown). Although the particular cell sections recorded for Figs. 4C, G, I and J were taken at the level of the cell body, so that the neurites are not apparent, other sections (not shown) revealed that the membrane-associated staining obtained using each of the anti-channel subunit antisera was localised to both the cell body and the neurites. An example of such localisation is apparent in Fig. 4D for a section stained with anti-α_2(469–483) serum.

Although the anti-α_2(469–483) serum strongly stained unpermeabilised cells, as shown in Fig. 4D, permeabilisation enhanced the immunostaining of the α_2 subunit. Quantification of the membrane-associated staining showed an increase from 106 ± 30 arbitrary units to 206 ± 39 arbitrary units (mean ± S.E.M., n = 6) following permeabilisation with 0.02% Triton X-100. In both detergent-treated and untreated cells immunostaining of similar apparent intensity was obtained following incubation with anti-α_2(1–15) serum and also using anti-α_2/δ(933–951) serum.

The ability of each of the three antibodies against residues 1–15, 469–483 and 933–951 of the α_2/δ primary translation product to label intact DRGs indicates that these regions, namely the N-termini of both the α_2 and δ polypeptides and also the sequence α_2(469–483), located on the predicted cytoplasmic loop in the model of Ellis and co-workers [11], are all extracellular. Thus, although the latter loop contains a consensus sequence for cAMP-dependent phosphorylation around serine 477, this is presumably not available for phosphorylation under physiological conditions. The extracellular locations of both the N-terminus of α_2 and the region containing residues 469–483, established in the present study, suggest that residues 422–445, which are predicted to form a membrane-spanning helix, are in fact unlikely to traverse the membrane. Because the N-terminus of the δ subunit was found to be extracellular, the C-terminus of α_2 must also be extracellular. Therefore the second putative transmembrane helix in the latter subunit, com-

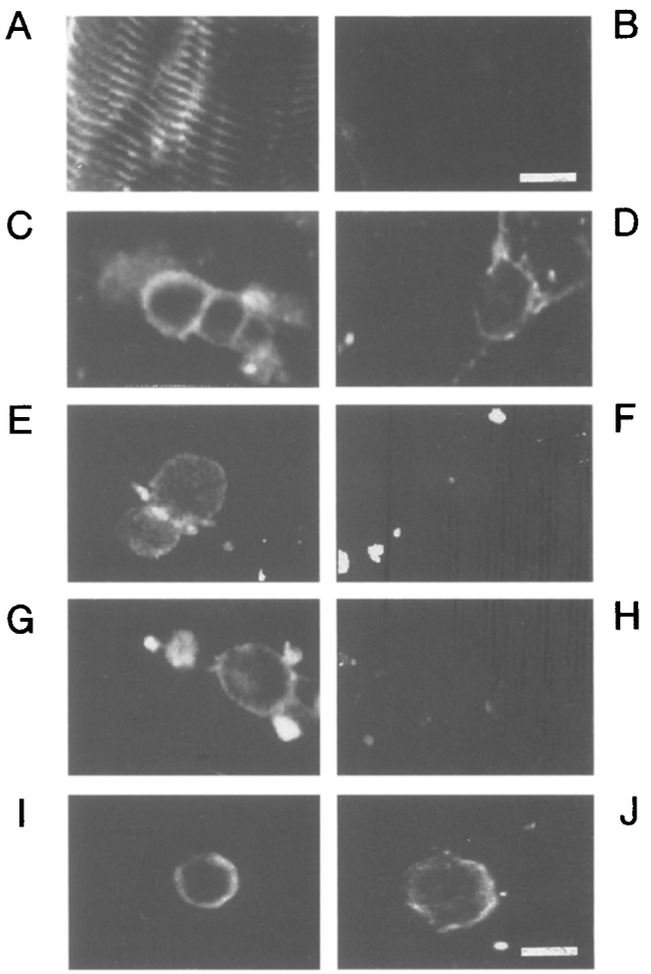

Fig. 4. Immunofluorescent staining of L-type calcium channels. Longitudinal, unfixed rat skeletal muscle sections were stained using either affinity purified anti-α_2(469–483) antibody (A) or control rabbit IgG (B). Panels C and D are representative of anti-α_2(469–483) antiserum staining of detergent permeabilised and intact DRGs respectively. Panels E and F show anti $G\alpha_o$ affinity purified antibody staining of detergent permeabilised and intact DRGs respectively. Panels G and H show anti-calcium channel β-subunit antiserum staining of detergent permeabilised and intact DRGs respectively. Panels I and J show immunostaining of intact DRGs obtained using anti-α_2(1–15) and anti-α_2/δ(933–951) antisera, respectively. The images shown are 1 μm confocal sections. The scale bar for panels A and B is 10 μm and for panels C–J is 20 μm. Panels C–J are representative of results obtained for at least four separate preparations of DRGs, each of which yielded results essentially identical to those shown for all cells examined.

prising residues 895–919 (of the rabbit protein) [11], is also unlikely to cross the membrane. Our findings therefore provide additional direct evidence for the hypothesis of Jay and coworkers [13] that the α_2 subunit is wholly extracellular, and is attached to the membrane via its disulphide linkage to the δ-subunit. However the finding that membrane permeabilisation enhanced immunostaining of the α_2 subunit by anti-α_2(469–483) serum in DRGs suggests that this antigenic domain is partially occluded by membrane association.

Acknowledgements: This work was supported by the Wellcome Trust, and by a Royal Free Hospital School of Medicine Junior Research Fellowship held by K.B. V.C. is in receipt of a Wellcome Prize Studentship.

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