Antibodies against the beta subunit of voltage-dependent calcium channels in Lambert-Eaton myasthenic syndrome

Cécile Raymond, Bichet Delphine, Iborra Cecile, Martin-Moutot Nicole, Seagar Machael, Michel Dewaard

Pergamon

PII: S0306-4522(98)00378-9

Neuroscience Vol. 90, No. 1, pp. 269–277, 1999

0306–4522/99 $19.00+0.00

ANTIBODIES AGAINST THE β SUBUNIT OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN LAMBERT–EATON MYASTHENIC SYNDROME

C. RAYMOND, D. WALKER, D. BICHET, C. IBORRA, N. MARTIN-MOUTOT, M. SEAGAR and M. DE WAARD*

INSERM U464, Laboratoire de Neurobiologie des Canaux Ioniques, Institut Fédératif Jean Roche, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13916, Marseille cedex 20, France

Abstract—Lambert–Eaton myasthenic syndrome is an autoimmune disease that impairs neuromuscular transmission. Several studies suggest that neurotransmitter release is reduced by an immune response directed against the calcium channel complex of nerve terminals. The immunoglobulin G fractions from Lambert–Eaton myasthenic syndrome patients immunoprecipitate solubilized neuronal N- and P/Q-type channels and in certain cases brain, skeletal and cardiac muscle L-type channels [El Far O. *et al*. (1995) *J. Neurochem*. **64,** 1696–1702; Lennon V. A. and Lambert E. H. (1989) *Mayo Clin. Proc*. **64,** 1498–1504; Sher E. *et al*. (1989) *Lancet* **ii,** 640–643; Suenaga A. *et al*. (1996) *Muscle Nerve* **19,** 1166–1168]. These channel immunoprecipitation assays are considered as useful for the diagnosis of this syndrome. In this study, we demonstrate that two predominant neuronal voltage-dependent calcium channel β subunits (β_3 and β_4, of mol. wt 58,000) are general targets of Lambert–Eaton myasthenic syndrome autoantibodies. Of 20 disease sera tested, 55% were able to immunoprecipitate ^{35}S-labeled β subunits. All five patients affected with small-cell lung carcinoma were positive for the β-subunit immunoprecipitation assay. Interestingly, only a fraction of the β-subunit-positive sera was also able to immunoprecipitate N- and P/Q-type channels, suggesting that several of the β-subunit epitopes are masked in native channels. In accordance with this observation, we found that several β-positive sera were able to prevent the interaction between calcium channel α_1 and β subunits *in vitro*. In cases where sera were able to immunoprecipitate β subunits, N- and P/Q-type channels, the immunoprecipitation of both channel types was either partially or entirely mediated by β-subunit antibodies.

Our results suggest that assays based on the immunoprecipitation of β subunits can be used as an additional test to assist in the diagnosis of Lambert–Eaton myasthenic syndrome. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: LEMS, calcium channels, β subunits, interaction sites, transmitter release, neuromuscular junction.

Lambert–Eaton myasthenic syndrome (LEMS) is a human autoimmune disorder[17] characterized by proximal muscle weakness and autonomic neuropathies. In 60% of cases, the disease is associated with small-cell lung carcinoma (SCLC).[26] Ataxia and subacute cerebellar degeneration[4] have also been reported in LEMS patients. Muscle weakness seems to be a consequence of reduced neurotransmitter release. At the neuromuscular junction, LEMS antisera decrease the number of active zones. Presynaptic intramembrane particles, thought to be voltage-dependent calcium channels (VDCCs) observed in freeze fracture views of the neuromuscular junction, are also disorganized.[12] Further evidence that VDCCs are targets of LEMS antibodies comes from biochemical data and functional analysis of the effects of antibodies on Ca^{2+} currents. Immunoglobulin G (IgG) fractions from patients with LEMS immunoprecipitate [^{125}I]ω-conotoxin GVIA (ω-GVIA)-labeled N-type channels.[19,20,31] LEMS IgGs also immunoprecipitate [^{125}I]ω-conotoxin MVIIC (ω-MVIIC)-labeled P/Q-type channels.[25] Both channels play important presynaptic functions in controlling the release of neurotransmitters,[33,36,38] therefore demonstrating their pertinence to the disease. LEMS IgGs also seem to inhibit P/Q-type Ca^{2+} currents in RINm5F cells[22] and to reduce the number of VDCCs present in cultured SCLC cell lines,[28] both effects possibly explaining the reduced acetylcholine release responsible for muscle weakness. VDCCs seem to be a specific target of LEMS antibodies, as other voltage-dependent channels are spared.[16]

No reliable model is available to explain the functional effects of LEMS autoantibodies on VDCC function. Current views hold that the antibodies limit Ca^{2+} influx through the channel, with a concomitant

*To whom correspondence should be addressed.

Abbreviations: AID, α_1 interaction domain; BID, β interaction domain; GST, glutathione-*S*-transferase; ω-GVIA, ω-conotoxin GVIA; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IgG, immunoglobulin G; LEMS, Lambert–Eaton myasthenic syndrome; ω-MVIIC, ω-conotoxin MVIIC; SCLC, small-cell lung carcinoma; VDCC, voltage-dependent calcium channel.

reduction in acetylcholine release.[16] It was indeed found that P/Q-type channel activity and exocytosis were blocked by LEMS IgGs in SCLC cells.[37] However, LEMS IgGs also appear to block L-type currents in NG 108 15 cells[27] and T-type channels in murine dorsal root ganglion neurons,[13] two channel types not involved in transmitter release at the neuromuscular junction. Clearly, the important question in LEMS is the antibody selectivity that ensures a selective depression of transmitter release at the neuromuscular junction via P/Q-type channels. The observation that several other channel types may be affected by LEMS IgGs would not ensure the required pathogenetic specificity of action of LEMS antibodies. It is therefore essential to analyse several other routes of potential mechanisms of presynaptic inhibition than a general blocking effect on channel gating.

Purification of two high-voltage-activated calcium channels, the skeletal muscle L-type channel[11] and the brain N-type channel,[39] has taught us a great deal about their molecular organization. VDCCs contain at least three subunits: the α_1 subunit, the ionic pore of the channel, the β subunit, which has a cytoplasmic localization, and the glycosylated $\alpha_2\delta$ subunit, an integral membrane protein with a large extracellular domain which, like β, is associated with α_1. The potential for high-threshold calcium channel epitope diversity is great,[2] since there are now six genes known to code for α_1 subunits, four for β and one for $\alpha_2\delta$. Potential LEMS epitope diversity is in fact even greater, because presynaptic VDCCs (N and P/Q types) are known to interact with proteins involved in exocytosis, which are relevant to the release of acetylcholine, namely SNAP-25, syntaxin and synaptotagmin. In fact, autoimmunity against synaptotagmin[19,34,35] has also been proposed as the basis for LEMS (but see Ref. 14). Therefore, epitope multiplicity in LEMS requires investigation for a better understanding of the pathology and mechanisms of action of IgG in presynaptic inhibition.

EXPERIMENTAL PROCEDURES

Patients

We used the sera of 20 LEMS patients (S1–S20) chosen randomly from various hospitals in France. The diagnosis of LEMS was based on clinical signs and electromyographic evidence. Thirteen patients had no detected neoplasm, five patients had SCLC (S5, S6, S9, S14 and S19) and two patients were undefined. Immunosuppressive therapy was followed by four patients (S1, S6, S10 and S13).

Complementary DNA clones

Full-length rabbit β_3 (GenBank accession code M88751) and rat brain β_4 (GenBank no. L02315) were subcloned into pGEM-3 and their synthesis was directed by a T7 promoter. The $\alpha_2\delta_b$ (GenBank no. M86621) cDNA was subcloned into pcDNA3.

In vitro *translation*

Calcium channel subunits were synthesized *in vitro* using the TNT™ kit of Promega. Proteins were labeled by the incorporation of [^{35}S]methionine. After synthesis, non-incorporated [^{35}S]methionine was eliminated by a Sephadex G-25M exclusion column (Pharmacia).

Immunoprecipitation assays

The immunoprecipitation of rat brain N-type channels was performed as described previously.[1] In brief, crude synaptosome fraction from rat brain (0.3 mg of protein/ml) was incubated overnight at 4°C with 200 pM [^{125}I]ω-GVIA in buffer A (25 mM Tris, 150 mM NaCl, pH 7.4 HCl). Membranes were recovered by centrifugation and solubilized in buffer A supplemented with 1.5% Triton X-100. Insoluble materials were removed by centrifugation, whereas aliquots of the supernatant (containing 6–8 fmol of toxin-labeled N-type channels) were incubated with 5 µl of serum or less for 4 h at 4°C in buffer B (sodium phosphate buffer, 0.1% Triton X-100, pH 7.4). The immune complexes were then immobilized by the addition of 50 µl protein A–Sepharose Fast Flow, washed twice by resuspension in buffer B and measured by γ counting.

The immunoprecipitation of P/Q-type calcium channels was performed as described by Martin-Moutot *et al.*[24] Rat cerebellar synaptosomes were solubilized in buffer C (320 mM sucrose, 10 mM HEPES, pH 7.4 NaOH, 2% digitonin). The soluble fraction was recovered by a 1-h centrifugation at 200,000 × *g* and the protein concentration of the supernatant determined using the Lowry method (Biorad). The soluble fraction (0.13 mg/ml) was labeled for 2 h with 0.2 nM [^{125}I]ω-MVIIC at 4°C in buffer D (320 mM sucrose, 7.5 mM NaCl, 2.5 mM Tris, 10 mM HEPES, pH 7.4). [^{125}I]ω-MVIIC-labeled receptor (7.7 fmol) was incubated for 3 h at 4°C with 5 µl of sera or less in a final volume of 200 µl. Immunoprecipitation was triggered by adding anti-human goat antiserum. Non-specific immunoprecipitation was performed in the presence of excess cold (× 1000) ω-MVIIC.

Immunoprecipitation of *in vitro* translated calcium channel subunits was performed by overnight incubation at 4°C of 2 µl translation lysate, 10 µl serum or less and 40 µl protein A–Sepharose beads in buffer B (final volume 250 µl). The final [^{35}S]β and [^{35}S]$\alpha_2\delta$ protein concentration was 1–3 pM in the assay. The immune complexes immobilized on the protein A–Sepharose beads were washed four times with 1 ml buffer B to eliminate unbound ^{35}S-labeled protein, transferred to scintillation vials and measured by β counting.

All immunoprecipitation assays were performed over the same period of time and at submaximal IgG concentrations, as determined by preliminary saturation analysis. In a few cases, absence of immunoprecipitation by LEMS sera was confirmed by the use of protein L instead of protein A, because of its wider range of human immunoglobulin binding.

α_1–β binding assay

The effects of LEMS sera were also tested on the association between a glutathione-*S*-transferase (GST) fusion protein expressing the major α_1 interaction site (defined as AID: α_1 interaction domain) and *in vitro* translated β subunit. This interaction was performed as described previously.[8] In brief, 1–2 µl lysate containing [^{35}S]β_3 were incubated for 30 min with either control or LEMS sera. Next, 1 µM purified GST–AID_A (AID sequence of the α_{1A} subunit, a constituent of P/Q-type channels) was coupled to 40 µl glutathione–agarose beads in 200 µl Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.4 HCl). Control [^{35}S]β_3 lysate or lysate preincubated with either control or LEMS sera were then added to the AID_A–GST–glutathione–agarose beads complex for 2–3 h. The beads

were then washed four times with Tris-buffered saline to remove unbound [^{35}S]β_3 and the bound radioactivity was determined by β counting.

Antisera production

The full-length β_3 subunit (GenBank accession code M88751) was subcloned in frame into the BamH1 and EcoR1 sites of the pGEX2TK vector and the resultant GST fusion protein was purified according to standard protocols. Next, polyclonal antibodies were raised in rabbits by injection of 100 μg purified GST–β_3 fusion protein after harvesting 12 ml of non-immune serum (control). After boosting the immune response of the rabbit three times, the rabbit was bled and the presence of β_3 antibodies was tested by enzyme-linked immunosorbent assay against GST–β_3, immunoblots of brain synaptosomes and immunoprecipitation of *in vitro* translated [^{35}S]β_3 subunit.

Calcium channel antagonists

ω-MVIIC was a generous gift from Dr Kazuki Sato. ω-GVIA was obtained from the Peptide Institute (Osaka, Japan). Iodination of both toxins was performed with Iodogen ([^{125}I]ω-MVIIC) or lactoperoxidase ([^{125}I]ω-GVIA), and mono-iodinated derivatives were purified by high-performance liquid chromatography, as described previously.[23] The specific activity of both toxins was 2600 c.p.m./fmol.

RESULTS

Several experimental data have unambiguously identified N- and P/Q-type channels as the channels controlling exocytosis and synaptic transmission at nerve terminals. We therefore sought to confirm that both channels were indeed potential targets of LEMS sera (Fig. 1A). The results demonstrate that eight sera out of 20 tested (40%) were positive for N-type channel immunoprecipitation. The fraction of P/Q-type channels immunoprecipitated by LEMS sera was even greater, with 14 positive sera out of 20 (70%). These results confirm previous findings that the immunoprecipitation of P/Q-type channels may be a better diagnostic test for LEMS. Next, we compared the efficacies of the sera that had been used for N- and P/Q-type channel immunoprecipitation. The data demonstrate that, of 20 LEMS sera tested, two exclusively immunoprecipitated N-type channels, eight immunoprecipitated P/Q-type channels only, six immunoprecipitated both channels and four immunoprecipitated neither of these two channel types (Fig. 1B). The results seem to indicate that the majority of N-positive sera are generally also P/Q-positive, making this last channel type the major target of LEMS antibodies. Combined N and P/Q immunoprecipitation assays revealed 16 positive cases out of the 20 (80%) diagnosed LEMS patients. In the case of the four non-immunoprecipitating sera, it is proposed that the autoantibodies may either recognize other channel types involved in transmitter release (i.e. the R-type channel), proteins relevant to exocytosis but not associated with calcium channels, or simply recognize non-immunoprecipitating calcium channel epitopes.

In a first step to address the epitope diversity in LEMS, we investigated the ability of the auto-

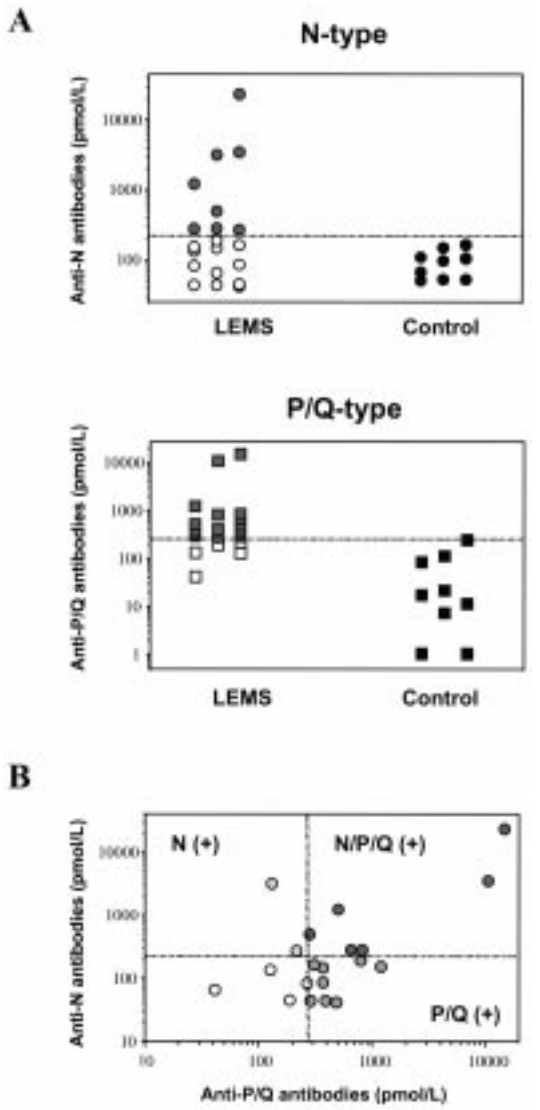

Fig. 1. (A) Immunoprecipitation assay for anti-N-type calcium channel autoantibodies (upper panel) and for anti-P/Q-type calcium channel autoantibodies (lower panel). N-type calcium channels were labeled with [^{125}I]ω-GVIA, whereas P/Q-type calcium channels were labeled with [^{125}I]ω-MVIIC. The dotted line represents the threshold for positivity as defined as a value greater than three standard deviations above the mean for the healthy controls (n=9 for N- and P/Q-channel types). N- and P/Q-type channel immunoprecipitation thresholds for positivity were 213 and 276 pmol/l, respectively. Gray filled symbols are signs of positivity. (B) Correlation between N- and P/Q-type calcium channel immunoprecipitation assays. Sera tested were S1–S20.

antibodies to immunoprecipitate calcium channel auxiliary subunits. High-voltage-activated calcium channels are composed of at least three subunits, the α_1 subunit which forms the ionic pore, and two subunits that modulate channel activity and biosynthesis, β and $\alpha_2\delta$. It is therefore likely that alterations in channel regulation may occur via binding of LEMS autoantibodies to one or both ancillary

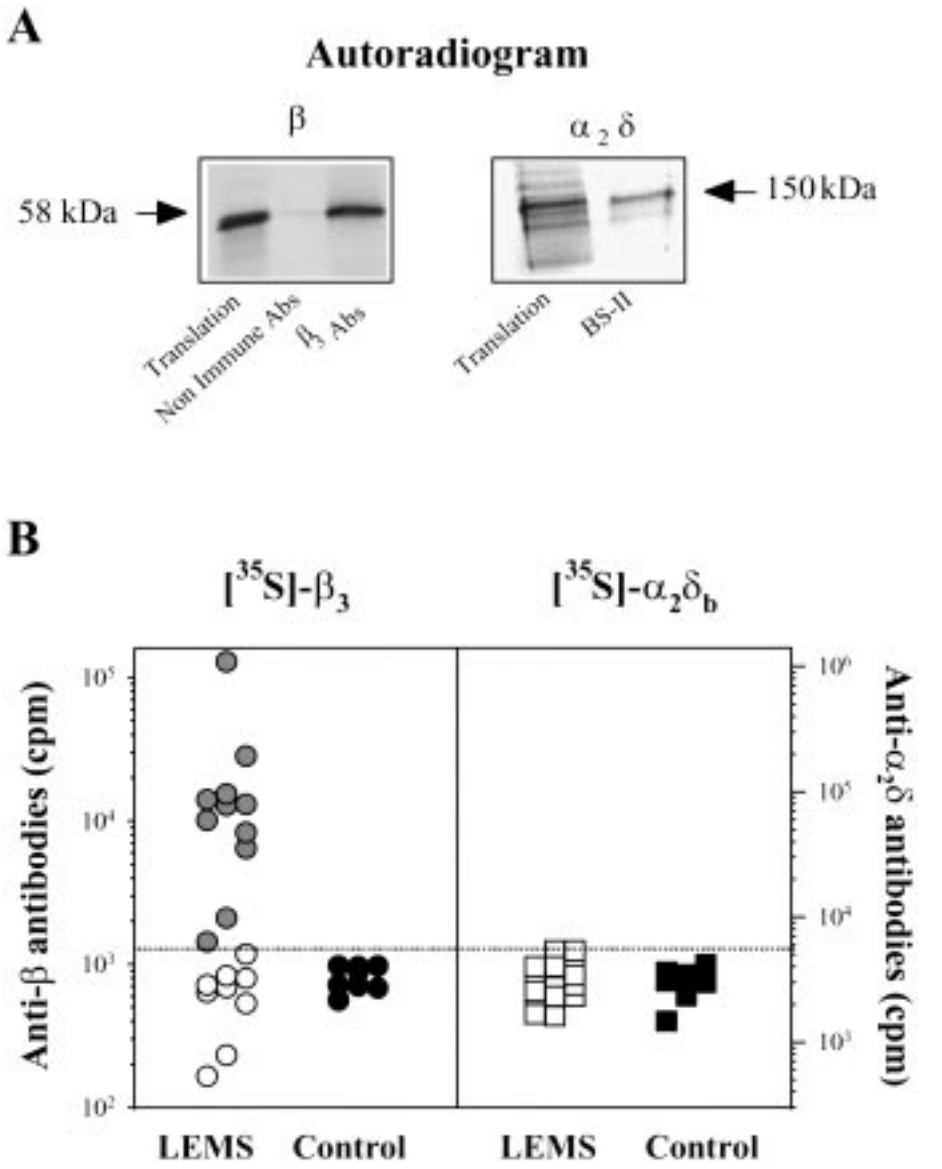

Fig. 2. (A) *In vitro* translation, precipitation and glycosylation of calcium channel auxiliary subunits. (Left) Lane 1: 5 µl reticulolysate containing $[^{35}S]\beta_3$; lanes 2 and 3: $[^{35}S]\beta_3$ immunoprecipitated by non-immune serum and polyclonal anti-GST–β_3 antibodies, respectively. (Right) Lane 1: 5 µl *in vitro* translated $[^{35}S]\alpha_2\delta_b$ in the presence of canine microsomal membranes; lane 2: glycosylated $[^{35}S]\alpha_2\delta_b$ precipitated by *Bandeiraea simplicifolia*-II–agarose beads. (B) Immunoprecipitation of $[^{35}S]\beta_3$ (S1–S20, circles) and $[^{35}S]\alpha_2\delta_b$ (S1–S19, squares) by LEMS sera. As for N- and P/Q-type channels, positivity is defined as the mean of immunoprecipitation by control sera ± 3 S.D. ($n=9$). $[^{35}S]\beta_3$ and $[^{35}S]\alpha_2\delta_b$ immunoprecipitation thresholds were 1301 and 5312 c.p.m., respectively (dotted lines). Gray filled circles denote positive signals (S4–S10, S14, S16, S19 and S20).

subunits, in addition to the α_1 channel protein. Figure 2A shows *in vitro* translated [^{35}S]methionine-labeled β_3 and $\alpha_2\delta_b$ calcium channel subunits, two isoforms highly expressed in the brain and other neuronal tissues. Since translation of $\alpha_2\delta_b$ was performed in the presence of canine microsomal membranes to favor glycosylation, a major glycosylated size form of mol. wt 150,000 was detected. As expected, $[^{35}S]\beta_3$ could be immunoprecipitated by purified rabbit anti-β_3 IgGs and the carbohydrate chains of $[^{35}S]\alpha_2\delta_b$ ensured recognition by *Bandeiraea simplicifolia*-II, a lectin coupled to agarose beads. Having confirmed the identity of the translated proteins, we tested the ability of various LEMS sera to immunoprecipitate both $[^{35}S]\beta_3$ and $\alpha_2\delta_b$ subunits (Fig. 2B). The results demonstrate that a significant proportion of LEMS sera immunoprecipitated $[^{35}S]\beta_3$ protein (11 of 20; 55%), whereas none of the tested sera immunoprecipitated $[^{35}S]\alpha_2\delta_b$ protein ($n=19$). In addition, we found that $[^{35}S]\beta_4$ was also immunoprecipitated by the same set of LEMS sera (data not shown), suggesting that conserved

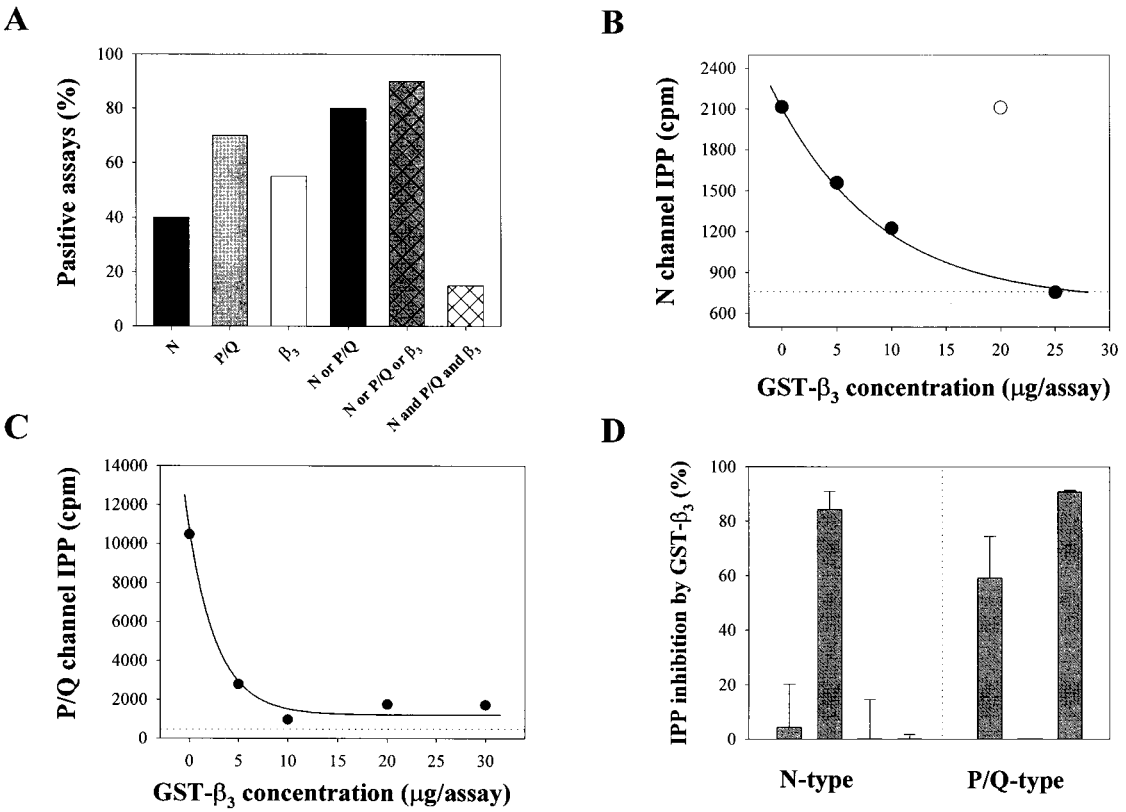

Fig. 3. (A) Percentage of sera positive for N-type channels, P/Q-type channels and β_3 subunit immunoprecipitation. Data were gathered from the same pool of 20 sera, for which all three immunoprecipitations were performed in parallel. Percentage of sera positive for N or P/Q immunoprecipitation, N, P/Q or β_3 immunoprecipitation, and N, P/Q and β_3 immunoprecipitation is also shown. (B) Displacement by purified β_3–GST of N-type channel immunoprecipitation by S10. Open circle shows the absence of displacement by 20 μg purified GST used as a control. Data were fitted by the exponential equation $y=\max\cdot\exp(-ax)+b$, where max = 1430 c.p.m., $a=0.105$ μg and $b=680$ c.p.m. Dotted line: control serum immunoprecipitation; IPP: immunoprecipitation. (C) As in B, but displacement by GST–β_3 of P/Q-type channel immunoprecipitation by S10. Control serum immunoprecipitation at 490 c.p.m. Fit yielded max = 9532 c.p.m., $a=0.3411$ μg and $b=1200$ c.p.m. (D) Inhibition of N- and P/Q-type channel immunoprecipitation by 30 μg GST–β_3 for N-β_3-positive sera ($n=4$; S6, S10, S14 and S19) and P/Q-β_3-positive sera ($n=3$; S10, S14 and S19).

β-subunit sequences were recognized by the antibodies. Control experiments also demonstrated the specificity of β-subunit precipitation, as [^{35}S]β_3 was immunoprecipitated by neither control sera (Fig. 2B), nor sera from patients with amyotrophic lateral sclerosis (data not shown). Interestingly, it was found that the sera isolated from LEMS patients with SCLC all immunoprecipitated β subunits, an observation that could not be extended to the precipitation of N or P/Q channel types (data not shown). However, certain patients with no detected neoplasm also produced anti-β-subunit antibodies.

In the same set of 20 sera, we directly compared the immunoprecipitation of N- and P/Q-type channels and β_3 subunits (Fig. 3A). The results suggest that the β_3-subunit assay reveals a significant fraction of positive LEMS sera, lower than the P/Q-type channel assay but higher than the N-type channel assay. Combining both N- and P/Q-type channel assays results in an improvement in sensitivity (80% of cases detected), whereas combining all three assays revealed $n=18$ out of 20 (90%). We conclude that the β_3 assay does not represent an alternative to the N- and P/Q-type channel assays, although it seems more sensitive than the immunoprecipitation of N-type channels. However, it slightly increases the percentage of seropositive LEMS patients when combined with the precipitation of N- and P/Q-type channels. In fact, β_3-subunit-positive sera were not necessarily all positive for both N- and P/Q-type channel immunoprecipitation. We found that only three sera out of 20 (15%) were positive in all three tests, indicating that N- and P/Q-type channel immunoprecipitation does not systematically rely on recognition of β-subunit epitopes. In two LEMS cases, significant [^{35}S]β_3 immunoprecipitation occurred without concomitant N- or P/Q-type channel immunoprecipitation. In other cases, where the sera were positive in all three tests, we determined to what extent β_3 antibodies were indeed responsible for N- and P/Q-type

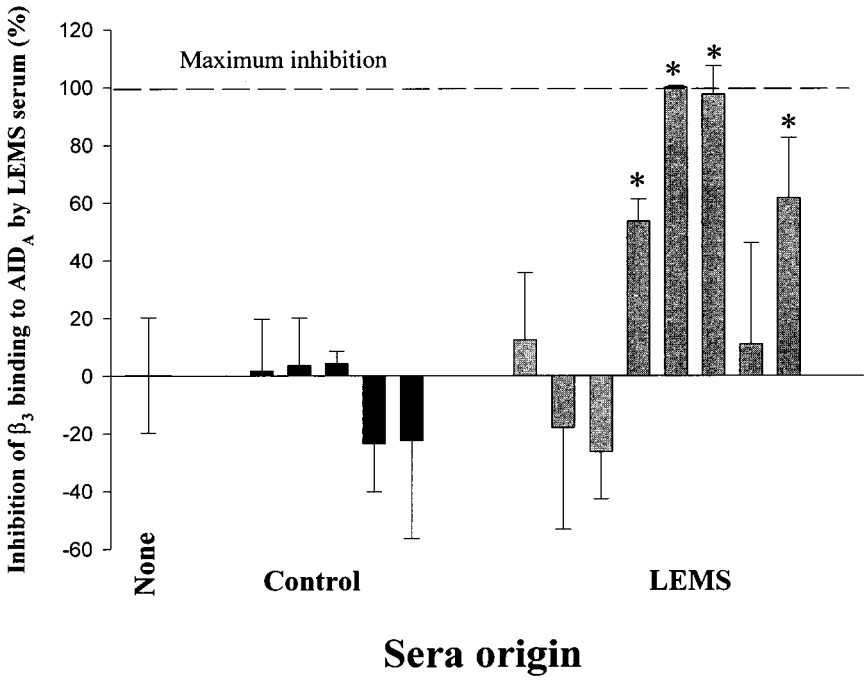

Fig. 4. Effect of LEMS β_3 autoantibodies on α_1–β association (n=8; S6–S8, S10, S14, S17, S19 and S20). Results are expressed as percentage reduction in GST–AID_A association to [^{35}S]β_3. GST–AID_A concentration is 1 μM. Maximum association is defined as the binding of GST–AID_A to [^{35}S]β_3 in the absence of autoantibodies. The effects of control sera on the association are also shown (n=5).

channel immunoprecipitation. To address this question, we preincubated these sera with various concentrations of purified β_3–GST fusion protein. The fusion protein and the autoantibodies directed against the β_3 sequence were then removed by adding glutathione–agarose beads to the sera, followed by centrifugation. The supernatants containing the sera depleted of β_3 antibodies to various extents were then tested in N-type (Fig. 3B) and P/Q-type (Fig. 3C) channel immunoprecipitation. The case of one serum (S10) is illustrated in which addition of 30 μg of GST–β_3 totally inhibits N- and P/Q-type immunoprecipitation (Fig. 3). In contrast, the addition of control GST had no effect on the immunoprecipitation. In this example, the immunoprecipitation of both N- and P/Q-type channels seems to rely solely on the presence of anti-β-subunit antibodies in the sera. Figure 3D summarizes similar studies on various other sera that were either positive in N-type channel and β_3-subunit assays (n=4) or positive in P/Q-type channel and β_3-subunit assays (n=7). In only one case out of four positive β_3 sera tested did preincubation with GST–β_3 totally inhibit N- or P/Q-type channel immunoprecipitation. In other cases, there was either a partial effect or no effect at all, suggesting that in most cases the immunoprecipitation of both N- and P/Q-type channels also relies on other associated proteins (SNARE proteins, for instance) or channel epitopes.

To understand how β_3-positive sera could be N- and/or P/Q-type negative, we tried to determine whether the β_3 epitopes recognized by some of the LEMS sera were situated at sites of interaction with other proteins. On the β subunit, one such site has been characterized, the BID (β subunit interaction domain), involved in the attachment of β to the α_1 channel.[9] Figure 4 demonstrates that sera preincubated with *in vitro* translated [^{35}S]β_3 subunit were indeed able to prevent the association of the β subunit with a GST fusion protein expressing the AID site (attachment site on the α_1 subunit; n=4 of 8). These data may explain why several LEMS sera contain β-subunit-targeted antibodies, but do not immunoprecipitate N- and P/Q-type channels.

DISCUSSION

We have confirmed the usefulness of N- and P/Q-type channel immunoprecipitation assays to detect autoantibodies in LEMS (Fig. 1). Taken together, we found that 80% of the sera had antibodies capable of immunoprecipitating either N- or P/Q-type channels (Fig. 3A). Interestingly, we found only two sera out of 20 positive for N-type but not for P/Q-type channel immunoprecipitation, suggesting that positive sera were mostly targeted towards unique P/Q channel epitopes or at least epitopes common to both N and P/Q channel types. These results are in accordance with the fact that neuromuscular transmission in normal human muscles is mostly mediated by P/Q-type channels, as indicated by their sensitivity to ω-agatoxin-IVA, a specific antagonist of this channel

type,[15,36] and the predominance of α_{1A} subunit localization in human motoneuron terminals.[7]

Channel immunoprecipitation by patients' sera also reveals that autoantibodies recognize multiple epitopes. This conclusion stems from the observations that (i) different channel combinations (N-type only, P/Q-type only, or both channel types) can be recognized by the various sera tested and (ii) 20% of the sera did not immunoprecipitate either N- or P/Q-type channels. In the latter case, however, one cannot rule out that the sera contain antibodies directed towards non-immunoprecipitating epitopes (disrupted by the solubilization process or accessible under certain functional conditions only) or towards epitopes masked by protein interaction sites. Several proteins have been postulated as possible targets of immune attack in LEMS. These can be divided into three types: (i) subunits of calcium channels (α_1, $\alpha_2\delta$ and β); (ii) proteins associated with calcium channels (SNARE proteins such as synaptotagmin, syntaxin and SNAP-25); and (iii) proteins not associated with calcium channels but relevant to the process of acetylcholine release at nerve terminals. As the third possibility falls beyond the scope of this article, we will not discuss it further. Previous antigen characterization has shown that a 58,000 mol. wt neuronal protein strongly reacts with purified LEMS IgGs and is relevant to channel immunoprecipitation by these antibodies.[19] Both synaptotagmin I and β subunits have been proposed as targets, as both the predominant neuronal β subunits (β_3 and β_4) and synaptotagmin have an apparent molecular weight of 58,000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Circumstantial immunoblot evidence has led to the proposal that the 58,000 mol. wt protein represents synaptotagmin.[19] This hypothesis is likely, since synaptotagmin is known to be associated with both N- and P/Q-type channels,[3] and antibodies directed against this molecule can immunoprecipitate these channel types. However, LEMS IgG fractions recognize a 55,000 mol. wt protein in skeletal muscle,[10] probably corresponding to the β_{1a} subunit. Also, we found that none of the sera used in this study was able to immunoprecipitate *in vitro* translated [^{35}S]synaptotagmin I (data not shown). The issue as to whether synaptotagmin can be a major target of LEMS autoantibodies is probably not yet settled, as at least 10 different genes code for this protein. In the El Far *et al*. study,[10] an additional 65,000 mol. wt protein was detected in the brain which seems unrelated to the β subunit. In any case, the present immunoprecipitation data on the β_3 subunit and the past cloning of a β subunit as the result of screening a human brain cDNA expression library with a LEMS serum[29] clearly demonstrate the importance of VDCC β subunits as a general target of LEMS IgG.

As β subunits are constituents of all high-voltage-activated calcium channels, we were curious to determine the role of β_3-targeted antibodies in N and P/Q channel immunoprecipitation. It was expected that the presence of antibodies directed against β_3 subunit would correlate with the immunoprecipitation of both N- and P/Q-type channels. This prediction was confirmed in only three cases out of 11. In the remaining cases, β_3-subunit-positive sera either immunoprecipitated (i) only N-channels ($n=1$ of 11), (ii) only P/Q-type channels ($n=5$ of 11) or (iii) none of these channels ($n=2$ of 11). Since β_3 is known to enter into the composition of both N- and P/Q-type channels,[21,30] these results cannot be interpreted on the basis of a specificity in β isoform recognition, particularly since LEMS sera did not distinguish β_3 from β_4 subunits (data not shown), another predominant neuronal β isoform entering into the subunit composition of both channels. These data instead suggest that the β-subunit epitopes recognized by the LEMS autoantibodies are masked by proteins interacting with β subunits. This assumption was partially confirmed by the demonstration that several LEMS sera were capable of significantly inhibiting β-subunit interaction with the AID sequence, an 18-amino-acid sequence of the α_{1A} subunit essential to the anchoring of β in the calcium channel complex, and to a role of β subunit in channel regulation and membrane targeting. In fact, one of the most effective sera in preventing the β-subunit interaction with α_1 was also one unable to immunoprecipitate N- or P/Q-type channels. These results suggest that the β epitope recognized can be entirely masked upon subunit association. This case clearly illustrates that it is not because a LEMS serum does not immunoprecipitate solubilized calcium channels that there are no antibodies directed against the channel in question. In our study, these β-subunit-positive and N- and P/Q-negative sera account for 10% of the LEMS patients.

The β subunit is a cytoplasmic protein and is unlikely to represent a primary target in the pathogenesis of LEMS. Therefore, the presence of anti-β-subunit antibodies in LEMS sera should be considered secondary to the disease process. There is precedence in the detection of cytoplasmic antigens in several other autoimmune diseases.[5] For instance, autoimmunity in Stiff-Man syndrome with breast cancer is targeted to the C-terminal region of human amphiphysin, another cytoplasmic protein.[6] As a secondary event, however, it is curious to realize that the β immunoreactivity presents several signs of specificity: (i) it is observed that the β subunit is considerably more immunoreactive than $\alpha_2\delta$, in spite of the existence of extensive extracellular domains in the latest; (ii) none of the sera recognized synaptotagmin I, also associated with an intracellular loop of α_{1A} and α_{1B}, and this in spite of its close molecular proximity to the β subunit; and, most importantly, (iii) several LEMS sera recognized β-subunit epitopes that are masked in native channels and that are not accessible after subunit assembly is completed *in vivo*. Any discussion on the role of β-subunit antibodies in LEMS is obviously purely speculative at this stage.

However, it should be noted that β subunits play essential roles in channel gating and biosynthesis.[9] All these effects require interaction between AID and BID, and it would be expected, though not proven, that the targeting of β subunits by LEMS antibodies could hamper channel biosynthesis and/or changes in channel properties. All reports so far of electrophysiological effects of LEMS IgGs on calcium currents conclude that there is a reduction in the number of functional VDCCs without affecting unitary channel properties.[16] These studies also suggest an indiscriminate block of high-voltage-activated channels (L, N and P/Q types) by LEMS IgG[13,27,37] compatible with a common antigen, such as the β subunit. The present demonstration that there are LEMS antibodies impairing BID function could support such a hypothesis. In light of these data, it will also be interesting to re-examine the immunoprecipitation of L-type channels by β-positive and β-negative LEMS sera and to determine whether immunoprecipitation of this channel type correlates with the presence of anti-β-autoantibodies in the serum.

CONCLUSIONS

Several laboratories have described serological assays for LEMS autoantibodies based on the immunoprecipitation of solubilized and toxin-labeled VDCCs.[18,20,25,31] The efficacy of these methods is variable and ranges from 50% to 90% depending on the research groups, the starting material being used, the number of sera tested and the patient's profile (presence or absence of SCLC). Evidence has been presented that tests based on the immunoprecipitation of the P/Q-type channel were more efficient than those based on the immunoprecipitation of N-type channels.[32] We have confirmed these results and have shown that positivity was greater in [^{125}I]ω-MVIIC receptor immunoprecipitation than in [^{125}I]ω-GVIA receptor immunoprecipitation. The immunoprecipitation of *in vitro* translated proteins also does not account for all cases of LEMS. However, our data indicate that it could be used as an additional aid in LEMS diagnosis, particularly for patients affected by SCLC. Obviously, the approach described here could be generalized to the study of other potential IgG LEMS targets, such as various synaptotagmins, syntaxin, SNAP-25 and synaptobrevin. Experiments are underway to detect antibodies against these proteins in LEMS sera.

Acknowledgements—Financial support was provided by the Association Française contre les Myopathies. Denise Walker is supported by a postdoctoral fellowship (Poste Vert) from the Institut National de la Santé et de la Recherche Médicale.

REFERENCES

1. Arsac C., Raymond C., Martin-Moutot N., Dargent B., Couraud F., Pouget J. and Seager M. (1996) Immunoassays fail to detect antibodies against neuronal calcium channels in amyotrophic lateral sclerosis serum. *Ann. Neurol.* **40,** 695–700.
2. 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. and Tsien R. W. (1994) The naming of voltage-gated calcium channels. *Neuron* **13,** 505–506.
3. Charvin N., Leveque C., Walker D., Berton F., Raymond C., Kataoka M., Shoji-Kasai Y., De Waard M. and Seagar M. (1997) Direct interaction of the calcium channel sensor protein synaptotagmin I with a cytoplasmic domain of the α_{1A} subunit of the P/Q-type calcium channel. *Eur. molec. Biol. Org. J.* **16,** 4591–4596.
4. Clouston P. D., Saper C. B., Arbizu T., Johnston I., Lang B., Newsom-Davis J. and Posner J. B. (1992) Paraneoplastic cerebellar degeneration. *Neurology* **42,** 1944–1950.
5. Dalmau J., Furneaux H. M., Gralla R. J., Kris M. G. and Posner J. B. (1990) Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer—a quantitative western blot analysis. *Ann. Neurol.* **27,** 544–552.
6. David C., Solimena M. and De Camilli P. (1994) Autoimmunity in Stiff-Man syndrome with breast cancer is targeted to the C-terminal region of human amphiphysin, a protein similar to the yeast proteins, Rvs167 and Rvs161. *Fedn Eur. biochem. Socs Lett.* **351,** 73–79.
7. Day N. C., Wood S. J., Ince P. G., Volsen S. G., Smith W., Slater C. R. and Shaw P. J. (1997) Differential localization of voltage-dependent calcium channel α_1 subunits at the human and rat neuromuscular junction. *J. Neurosci.* **17,** 6226–6235.
8. De Waard M., Witcher D. R., Pragnell M., Liu H. and Campbell K. P. (1995) Properties of the α_1–β anchoring site in voltage-dependent Ca^{2+} channels. *J. biol. Chem.* **270,** 12,056–12,064.
9. De Waard M., Gurnett C. A. and Campbell K. P. (1996) Structural and functional diversity of voltage-activated calcium channels. In *Ion Channels* (ed. Narahashi T.), pp. 41–87. Plenum, New York.
10. El Far O., Marquèze B., Leveque C., Martin-Moutôt N., Lang B., Newsom-Davis J., Yoshida A., Takahashi M. and Seagar M. (1995) Antigens associated with N- and L-type calcium channels in Lambert–Eaton myasthenic syndrome. *J. Neurochem.* **64,** 1696–1702.
11. Flockerzi V., Oeken H.-J., Hofmann F., Pelzer D., Cavalie A. and Trautwein W. (1986) Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. *Nature* **323,** 66–68.
12. Fukunaga H., Engel A. G., Lang B., Newsom-Davis J. and Vincent A. (1983) Passive transfer of Lambert–Eaton myasthenic syndrome with IgG from man to mouse depletes the presynaptic membrane active zones. *Proc. natn. Acad. Sci. U.S.A.* **80,** 7636–7640.
13. Garcia K. D. and Beam K. G. (1996) Reduction of calcium currents by Lambert–Eaton syndrome sera: motoneurons are preferentially affected, and l-type currents are spared. *J. Neurosci.* **16,** 4903–4913.
14. Hajela R. K. and Atchison W. D. (1995) The proteins synaptotagmin and syntaxin are not general targets of Lambert–Eaton myasthenic syndrome autoantibody. *J. Neurochem.* **64,** 1245–1251.

15. Kim Y. I., Longacher J. M. and Viglione M. P. (1993) ω-Agatoxin IVA blocks evoked quantal transmitter release at the mammalian neuromuscular junction. *Molec. Biol. Cell* **4 Suppl.,** A428.
16. Kim Y. I. and Neher E. (1988) IgG from patients with Lambert–Eaton syndrome blocks voltage-dependent calcium channels. *Science* **239,** 405–408.
17. Lambert E. H., Eaton L. M. and Rooke E. D. (1956) Defect of neuromuscular conduction associated with malignant neoplasm. *Am. J. Physiol.* **187,** 612–613.
18. Lennon V. A. and Lambert E. H. (1989) Autoantibodies bind solubilized calcium–ω-conotoxin complexes from small cell lung carcinoma: a diagnostic aid for Lambert–Eaton myasthenic syndrome. *Mayo Clin. Proc.* **64,** 1498–1504.
19. Lévêque C., Hoshino T., David P., Shoji-Kasai Y., Leys K., Omori A., Lang B., El Far O., Sato K., Martin-Moutôt N., Newsom-Davis J., Takahashi M. and Seagar M. J. (1992) The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert–Eaton myasthenic syndrome antigen. *Proc. natn. Acad. Sci. U.S.A.* **89,** 3625–3629.
20. Leys K., Lang B. and Newsom-Davis J. (1991) Calcium channel autoantibodies in the Lambert–Eaton myasthenic syndrome. *Ann. Neurol.* **29,** 307–314.
21. Liu H., De Waard M., Scott V. E. S., Gurnett C. A., Lennon V. A. and Campbell K. P. (1996) Identification of three subunits of the high affinity omega-conotoxin MVIIC-sensitive Ca^{2+} channel. *J. biol. Chem.* **271,** 13804–13810.
22. Magnelli V., Grassi C., Parlatore E., Sher E. and Carbone E. (1996) Down-regulation of non-L-, non-N-type (Q-like) Ca^{2+} channels by Lambert–Eaton myasthenic syndrome (LEMS) antibodies in rat insulinoma RINm5F cells. *Fedn Eur. biochem. Socs Lett.* **387,** 47–52.
23. Martin-Moutot N., Leveque C., Sato K., Kato R., Takahashi M. and Seagar M. (1995) Properties of omega conotoxin MVIIC receptors associated with alpha 1A calcium channel subunits in rat brain. *Fedn Eur. biochem. Socs Lett.* **366,** 21–25.
24. Martin-Moutot N., Charvin N., Leveque C., Sato K., Nishiki T., Kozaki S., Takahashi M. and Seagar M. (1996) Interaction of SNARE complexes with P/Q-type calcium channels in rat cerebellar synaptosomes. *J. biol. Chem.* **271,** 6567–6570.
25. Motomura M., Johnston I., Lang B., Vincent A. and Newsom-Davis J. (1995) An improved diagnostic assay for Lambert–Eaton myasthenic syndrome. *J. Neurol. Neurosurg. Psychiat.* **58,** 85–87.
26. O'Neill J. H., Murray N. M. F. and Newsom-Davis J. (1988) The Lambert–Eaton myasthenic syndrome. *Brain* **111,** 577–596.
27. Peers C., Lang B., Newsom-Davis J. and Wray D. W. (1990) Selective action of myasthenic syndrome antibodies on calcium channels in a rodent neuroblastoma × glioma cell line. *J. Physiol.* **421,** 293–308.
28. Roberts A., Perera S., Lang B., Vincent A. and Newsom-Davis J. (1985) Paraneoplastic myasthenic syndrome IgG inhibits $^{45}Ca^{2+}$ flux in a human small cell carcinoma line. *Nature* **317,** 737–739.
29. Rosenfeld M. R., Wong E., Dalmau J., Manley G., Posner J. B., Sher E. and Furneaux H. M. (1993) Cloning and characterization of a Lambert–Eaton myasthenic syndrome antigen. *Ann. Neurol.* **33,** 113–120.
30. Scott V. E. S., De Waard M., Liu H., Gurnett C. A., Venzke D. P., Lennon V. A. and Campbell K. P. (1996) Beta subunit heterogeneity in N-type Ca^{2+} channels. *J. biol. Chem.* **271,** 3207–3212.
31. Sher E., Gotti C., Canal N., Scopetta C., Evoli A. and Clementi F. (1989) Specificity of calcium channel autoantibodies in Lambert–Eaton myasthenic syndrome. *Lancet* **2,** 640–643.
32. Suenaga A., Shirabe S., Nakamura T., Motomura M., Tsujihata M., Matsuo H., Kataoka Y., Niwa M., Itoh M. and Nagataki S. (1996) Specificity of autoantibodies reacting with omega-conotoxin MVIIC-sensitive calcium channel in Lambert–Eaton myasthenic syndrome. *Muscle Nerve* **19,** 1166–1168.
33. Takahashi T. and Momiyama A. (1993) Different types of calcium channels mediate central synaptic transmission. *Nature* **366,** 156–158.
34. Takamori M., Hamada T., Komai K., Takahashi M. and Yoshida A. (1994) Synaptotagmin can cause an immune-mediated model of Lambert–Eaton myasthenic syndrome in rats. *Ann. Neurol.* **35,** 74–80.
35. Takamori M., Takahashi M., Yasukawa Y., Iwasa K., Nemoto Y., Suenaga A., Nagataki S. and Nakamura T. (1995) Antibodies to recombinant synaptotagmin and calcium channel subtypes in Lambert–Eaton myasthenic syndrome. *J. neurol. Sci.* **133,** 95–101.
36. Uchitel O. D., Protti D. A., Sanchez V., Cherksey B. D., Sugimori M. and Llinas R. (1992) P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proc. natn. Acad. Sci. U.S.A.* **89,** 3330–3333.
37. Viglione M. P., Oshaughnessy T. J. and Kim Y. I. (1995) Inhibition of calcium currents and exocytosis by Lambert–Eaton syndrome antibodies in human lung cancer cells. *J. Physiol.* **488,** 303–317.
38. Wheeler D., Randall A. and Tsien R. (1994) Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science* **264,** 107–111.
39. Witcher D. R., De Waard M., Sakamoto J., Franzini-Armstrong C., Pragnell M., Kahl S. D. and Campbell K. P. (1993) Subunit identification and reconstitution of the N-type Ca^{2+} channel complex purified from brain. *Science* **261,** 486–489.

(*Accepted* 4 *August* 1998)