changes in [Ca\(^{2+}\)], whereas a voltage pulse produces very rapid changes of [Ca\(^{2+}\)] close to the membrane. In Figure 1, however, values derived from ionomycin applications seem to indicate that for faster changes the dose-response curve should be extrapolated along the stippled curve. The value for secretion rates obtained during voltage pulses can be compared with this extrapolation to estimate the calcium level at the release site.

A more quantitative value, however, can be obtained from the model of Heinemann et al. [17] in which Ca-dependent rates for the release process were derived from data as shown in Figure 1, assuming a two-step process of vesicle supply into a release-ready pool (step 1) followed by the actual release (step 2). Assuming an initial size for the release-ready pool of 500 fF and secretion rates as derived from this model, it is seen that values for [Ca\(^{2+}\)] of between 2 and 3.5 μM are required at the release site. This is about a factor of 10 higher than increments of average cellular calcium recorded with fura-2 during such stimulation. The experimental rates (100-500 fF) and the size of the readily-releasable pool (500 fF) also imply that depletion of that pool occurs within 1-5 s when the cell is depolarized or when [Ca\(^{2+}\)] is increased by other experimental manipulations into the range of a few micromolar. Then, the overall reaction rate is no longer determined by the secretion process itself, but rather by the supply of new secretory granules.


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**A Lambert–Eaton myasthenic syndrome antigen associated with presynaptic calcium channels**

Michael J. Seagar,* Nicole Martin-Moutot,* Christian Leveque,* Oussama El Far,* Pascale David,* Béatrice Marquez,† Bethan Lang,† John Newsom-Davis,† Toshimitsu Hoshino‡ and Masami Takahashi‡

*INSERM U9016, Faculté de Médecine Secteur Nord, Bd. Pierre Dramard, 13326 Marseille Cedex 15, France, †Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K. and ‡Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan

**Introduction**

Lambert–Eaton myasthenic syndrome (LEMS) is a disorder of neuromuscular transmission first reported by Lambert and colleagues in 1956 ([1] and reviewed in [2]). It is associated in approximately 60% of cases with small cell lung carcinoma (SCLC) [3]. LEMS is characterized by muscle weakness and reduced tendon reflexes, and is often accompanied by autonomic dysfunction which results in dry mouth, sexual impotence and constipation. The compound action potential, recorded at the surface of an affected muscle following a single nerve stimulation, is abnormally small. However, high-frequency stimulation leads to a large potentiation in muscle response which is characteristic of the disease [1, 3]. Recordings from biopsied LEMS
muscle have demonstrated that the number of acetylcholine-containing quanta released per single nerve impulse is reduced [4]. These findings indicate that the abnormal muscle action potential is the consequence of diminished neurotransmitter release, and that LEMS therefore results from a defect in presynaptic mechanisms.

These electrophysiological characteristics can be transferred to mice by repeated injections of IgG fractions purified from the plasma of LEMS patients [5]. LEMS is therefore an autoimmune disease in which pathogenic antibodies target critical components of the excitation–secretion pathway. Experiments performed in several laboratories during the 1980s have provided strong evidence that LEMS autoantibodies reduce secretion by binding to voltage-gated calcium channels. Kim and Neher [6] used the patch-clamp technique to examine the effect of these autoantibodies on calcium currents in cultured bovine chromaffin cells. Incubation with LEMS IgG led to a significant decrease in calcium current amplitude without modifying the fundamental channel-gating properties. A maximum effect required several hours exposure to LEMS antibodies indicating that direct channel block was unlikely. Reduced calcium current is therefore the result of a decrease in the number of functional channels at the cell surface, possibly due to accelerated catabolism of channel proteins. Exocytosis was monitored indirectly using capacitance measurements as an index of increasing membrane area. LEMS IgG had no significant effect on this parameter when secretion was evoked by perfusing calcium into the cytoplasm via the recording pipette, which allows voltage-gated calcium entry to be bypassed [6]. LEMS antibodies do not therefore interfere directly with the exocytotic machinery in chromaffin cells, but downregulate the calcium channels responsible for triggering secretion.

The small size of nerve terminals has ruled out the use of this type of approach at the vertebrate neuromuscular junction. However, evoked release (quantal content of the endplate potential) can be measured as a function of extracellular calcium concentration. Experiments have shown that, in muscle from mice with passively transferred LEMS, more calcium was required to produce equivalent acetylcholine secretion [7]. These results are compatible with an immune-mediated reduction in the number of functional calcium channels at the presynaptic terminal.

SCLC cells also express calcium channels that are downregulated by LEMS IgG [8], providing a probable link between LEMS and cancer. An initial immune response is presumably directed against the tumour, and anti-calcium channel antibodies are produced that then cross-react with antigenically similar structures in synaptic terminals to produce the neurological syndrome.

Most neurons express several types of voltage-dependent calcium channel which have been classified according to their electrophysiological and pharmacological properties [9, 10]. The first calcium channel to be characterized biochemically was the L-type channel in skeletal muscle which was isolated as the receptor for the 1,4-dihydropyridine calcium channel antagonists. It has a hetero-oligomeric structure containing \( \alpha_1 \), \( \alpha_2\delta \), \( \beta \) and \( \gamma \) subunits [11]. The \( \alpha_1 \) subunit alone forms the calcium-conducting transmembrane pore and binds calcium antagonist drugs and toxins, while the other subunits appear to play a regulatory role. Molecular cloning has allowed the primary structure of these polypeptides to be elucidated, and skeletal muscle \( \alpha_1 \) probes have been used to isolate a family of homologous cDNAs from rat brain libraries [12].

Particularly useful tools for the study of neuronal calcium channels are the \( \omega \) conotoxins, a family of calcium-antagonist peptides isolated from the venoms of marine gastropods [13]. N-type calcium channels, which play a major role in excitation–secretion coupling, can be labelled with \([^{125}I]\omega\)-conotoxin GVIa (\([^{125}I]\omega\)-CgTx). The first biochemical assay for an anti-calcium channel antibody specificity in LEMS IgG was reported by Sher and collaborators [14]. They demonstrated that N-type calcium channels, solubilized from human neuroblastoma membranes and labelled with \([^{125}I]\omega\)-CgTx, were specifically immobilized by LEMS antibodies.

The aim of the research presented in this paper was to identify at a molecular level the LEMS antigen(s) associated with presynaptic N-type calcium channels. Our findings have some novel implications concerning the molecular mechanisms of synaptic vesicle docking at neurotransmitter release sites.

**Results and discussion**

**Which calcium channel-associated protein(s) bind(s) LEMS antibodies?**

Probing immunoblots of crude membrane preparations is evidently limited by the polyclonal nature of autoimmune IgG, patient to patient variability in immune response and the low density of \([^{125}I]\omega\)-CgTx receptors (< 1 pmol/mg of membrane
protein). The sensitivity of this approach, however, can be increased by receptor purification. A convenient source of presynaptic calcium channels for purification is rat brain synaptosomes. It was first necessary, however, to check that LEMS antibodies react with rat brain calcium channels. Synaptic N-type channels were labelled with $^{125}$I$\omega$-CgTx, extracted with Triton X-100 and incubated with LEMS IgG. Immune complexes were trapped on Protein A-Sepharose, and the presence of calcium channels ascertained by gamma counting. Approximately 80% of the $\omega$-CgTx receptors were precipitated by LEMS IgG, whereas less than 3% were recovered using healthy control IgG.

Solubilized $^{125}$I$\omega$-CgTx prelabelled calcium channels were purified by a two-step affinity procedure. Calcium channels bound to a column of Chelating Sepharose Fast Flow loaded with cobalt ions. After extensive washing, the column was eluted with EDTA. The eluted proteins were loaded onto a Heparin-Ultrogel column, and washed with 0.4 M NaCl. The $\omega$-CgTx receptor was then recovered by the addition of 0.8 M NaCl, with a yield of about 15% and a 300-fold purification. The purified proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane and probed with a range of human plasma or IgG using conventional Western blotting techniques. Initial experiments were carried out using a blind protocol in which 19 coded plasma and IgG samples from patients with EMS, other neurological or autoimmune diseases and from healthy volunteers were prepared in Oxford and then tested in Marseille. Five of these showed strong immunoreactivity with a 58 kDa protein band present in a partially purified calcium-channel preparation. When the code was broken these five samples were found to be from three LEMS patients (plasma and IgG from two patients and IgG from one patient). None of the control samples labelled this polypeptide, demonstrating that it is a specific LEMS antigen. However, samples from some LEMS patients also failed to show 58 kDa immunoreactivity. Subsequent experiments with a larger group of LEMS patients have demonstrated that only plasma or IgG that immunoprecipitate solubilized $^{125}$I$\omega$-CgTx receptors at high titre recognize the 58 kDa protein in immunoblots (Table 1). This LEMS antigen, however, is not the $\omega$-CgTx-binding polypeptide of the calcium channel. Covalent labelling studies with azidobenzoyl $^{125}$I$\omega$-CgTx derivatives [16–18] and more recently protein purification [19], have identified a 220–300 kDa protein that is presumably the $\alpha_1$ subunit of the N-type channel.

### Table I

<table>
<thead>
<tr>
<th>Western blots</th>
<th>Positive (&gt; 100 pM)</th>
<th>Equivocal (50–100 pM)</th>
<th>Negative (&lt; 50 pM)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>FA (1400)</td>
<td>CO (1400)</td>
</tr>
<tr>
<td></td>
<td>AL (733)</td>
<td></td>
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<tr>
<td><strong>Equivocal</strong></td>
<td>HN (602)</td>
<td></td>
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<tr>
<td></td>
<td>HU (65)</td>
<td>DU (58)</td>
<td>UN (2)</td>
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<td>GR (45)</td>
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<tr>
<td></td>
<td>AN (4)</td>
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Plasma samples from LEMS patients were assayed for their ability to immunoprecipitate $^{125}$I$\omega$-CgTx-labelled calcium channels (pM indicates pmol of $\omega$-CgTx receptor immunoprecipitated per litre of plasma; the individual titre for each patient is shown in parentheses) and to stain a 58 kDa band in Western blots of partially purified calcium channel [15].

**Is the 58 kDa protein a calcium-channel subunit?**

In view of the polyspecific nature of autoimmune IgG, it was first necessary to determine whether the same antibody population recognizes the 58 kDa protein in immunoblots and immunoprecipitates solubilized $^{125}$I$\omega$-CgTx receptors. The 58 kDa protein was therefore used as a support to affinity purify LEMS IgG. The purified IgG was subsequently shown to immunoprecipitate solubilized $^{125}$I$\omega$-CgTx receptors, indicating that LEMS antibodies immunoprecipitate the N-type calcium channel by binding to a tightly associated 58 kDa protein [15].

This LEMS antigen does not, however, appear to be a channel subunit. Western blotting experiments with crude neuronal homogenates using IgG from LEMS patients with a high titre of anti-$\omega$-CgTx receptor antibodies suggested the 58 kDa polypeptide was a major constituent of synaptic membranes. Sucrose density gradient centrifugation was therefore performed on solubilized rat brain synaptic membranes to compare the apparent
oligomeric size of the 58 kDa antigen with that of the $^{[251]}$o-CgTx-labelled calcium channel. This experiment demonstrated that most of the antigen sedimented at low velocity, and only a small fraction was associated with the more rapidly sedimenting calcium channel [15]. We therefore concluded that this LEMS antigen is not a channel subunit but a relatively major protein which can interact functionally with the channel.

**Identification of the LEMS antigen**

A monoclonal antibody (mAb) has been produced that has strikingly similar properties to LEMS IgG. In an attempt to obtain antibodies directed against N-type calcium channels SJL mice were immunized with chick brain synaptic membranes. Hybridomas were produced and screened for their ability to secrete antibodies that interact with $^{[251]}$o-CgTx receptors, using a protocol analogous to the LEMS immunoprecipitation assay described above. In this way mAb1D12 was generated. mAb1D12 immunoprecipitates calcium channel and recognizes a 58 kDa band in immunoblots of rat brain synaptic membranes [20]. Immunocytochemistry has shown the 1D12 antigen to be a fairly abundant constituent of synaptic regions of the central and peripheral nervous systems, that is present in both synaptic vesicles and plasma membrane [20].

In order to determine whether mAb1D12 and LEMS antigen do indeed recognize the same protein, two complementary experiments were performed. Firstly, LEMS IgG and mAb1D12 were used to probe Western blots of partially purified N-type calcium channel. Both labelled the same 58 kDa band. Secondly, 1D12 antigen was purified on a mAb 1D12-Sepharose 4B immunoaffinity column. LEMS IgG was shown to bind to this protein (Figure 1), whereas healthy human IgG did not [15]. The antigen in question was then identified by immunoscreening a rat brain Agt11 library with mAb1D12. Four overlapping clones were isolated, and the sequence obtained [15] was found to be virtually identical to nucleotides 577–2673 of synaptotagmin (p65), a synaptic vesicle membrane protein [21-25].

The molecular properties of synaptotagmin suggest that it is involved in exocytosis. It contains an internally repeated cytoplasmic sequence homologous to the C2 region of protein kinase C [22] and binds calmodulin [23]. It interacts with acidic phospholipids [22, 24] and calcium ions, suggesting that it may be a calcium receptor for transmitter release [25]. Direct functional evidence for synaptotagmin's physiological role is lacking. Recent work has demonstrated that synaptotagmin-deficient subclones of the pheochromocytoma cell line PC12 display normal catecholamine secretion in response to potassium depolarization [26]. Neurosecretory cell lines, however, cannot be considered as a relevant model for all types of neurotransmitter release, and the question as to whether synaptotagmin is necessary for rapid synaptic transmission at the neuromuscular junction still remains open.

**Discussion**

The results outlined in this paper point to two major conclusions. Firstly, synaptotagmin associates with N-type calcium channels. The protein remains associated with such channels during an affinity fractionation procedure that results in approximately 300-fold purification. Furthermore, a
monoclonal antibody selected initially for its ability to immunoprecipitate $[^{[125]}]\omega$-CgTx-labelled channels in fact binds to synaptotagmin. Secondly, antibodies from patients with LEMS, an autoimmune disease in which calcium-channel down-regulation results in defective transmitter release, also bind to synaptotagmin.

Our data are consistent with the hypothesis illustrated in Figure 2. They suggest a tight physical interaction between synaptotagmin, a synaptic vesicle transmembrane polypeptide, and the calcium channel, an oligomeric plasma membrane protein. Other proteins not shown in Figure 2 may be involved, including a 35 kDa polypeptide (synaptaxin, p35) recently identified independently in two laboratories, which is a synaptotagmin and N-type channel-associated protein [27, 28]. Interaction may play a role in docking synaptic vesicles at release sites. At the frog neuromuscular junction, N-type channels are localized exclusively at exocytotic active zones [29]. Association of synaptic vesicles with calcium channels would presumably site the calcium sensor protein(s) that trigger(s) exocytosis in a favourable zone rapidly accessible to calcium transients.

Docking at the active zone is probably a regulated process. Synaptotagmin carries several potential sites for modulation, including protein kinase C and Ca/calmodulin kinase II phosphorylation [20]. Membrane potential may conceivably play a role in docking as the calcium channel undergoes voltage-dependent conformational transitions corresponding to gating. One can imagine that these transitions may mask and unmask binding sites for other proteins at the cytoplasmic face. A precedent for direct interaction between a voltage-gated calcium channel at the plasma membrane and proteins in an intracellular compartment exists in skeletal muscle where excitation-contraction coupling is mediated by interaction between the T-tubule dihydropyridine receptor and the sarcoplasmic reticulum release channel.

If the autoantibodies directed against synaptotagmin are pathogenic in LEMS they presumably bind to an extracellularly orientated epitope. Following calcium-triggered exocytosis, the intravesicular N-terminal domain of synaptotagmin would be exposed at the cell surface. Circulating LEMS antibodies could then bind to a synaptotagmin–calcium channel complex. Multiple synaptotagmin isoforms are expressed in the nervous system [30–32]. Although the cytoplasmic domain is conserved, the N terminus, thought to bind LEMS IgG, is variable and glycosylated. An import-

Figure 2

A hypothetical model illustrating how the association between synaptotagmin and calcium channels may be involved in docking synaptic vesicles at the active zone.

After exocytosis, the intravesicular N-terminal domain of synaptotagmin would become accessible to LEMS IgG.
ant aim at present is to identify the synaptotagmin epitope(s) that bind LEMS IgG, and to determine whether antibodies directed against this motif can induce calcium channel downregulation and inhibition of rapid synaptic release in an animal model.

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