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Lambert–Eaton syndrome antibodies inhibit acetylcholine release and P/Q-type Ca^{2+} channels in electric ray nerve endings

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1. The types of voltage-dependent calcium channels (VDCCs) present in the cholinergic terminals isolated from the electric organ of the ray, *Narke japonica*, were characterized on the basis of their pharmacological sensitivity to specific antagonists. Inhibition of these channel types by autoantibodies from patients with the Lambert–Eaton syndrome (LES) was then studied to determine the specificity of the pathogenic IgG.

2. In normal untreated synaptosomal preparations, maximal doses of N- and P and/or Q-type Ca^{2+} channel antagonists, ω-conotoxin GVIA and ω-agatoxin IVA, inhibited depolarization-evoked ACh release by 47% and 43%, respectively. Calciseptine, an L-type VDCC antagonist, caused a 20% reduction in the release. This indicates that the exocytotic release process is predominantly mediated by N- and P/Q-type VDCCs.

3. LES IgG or sera caused an inhibition of ACh release by 39–45% in comparison with the control antibody-treated preparations. The ionomycin-induced ACh release, however, was not altered by the antibodies. Additionally, the same LES antibodies inhibited whole-cell calcium currents (I_{Ca}) in bovine adrenal chromaffin cells. Thus, the pathogenic antibodies exert their action on VDCCs present in the synaptosomes.

4. The efficacy of three Ca^{2+} channel antagonists in blocking ACh release was determined in preparations pretreated with LES IgG. ω-Agatoxin IVA produced only an additional 3–5% reduction in release beyond that obtained with LES antibodies. Despite the pretreatment with LES IgG, ω-conotoxin GVIA and calciseptine inhibited the release to nearly their control levels.

5. These results indicate that LES antibodies mainly downregulate P/Q-type Ca^{2+} channels which contribute to presynaptic transmitter release from the cholinergic nerve terminals of electric organ.

6. The present findings are consistent with the hypothesis that P/Q-type VDCCs at the neuromuscular junction are the target of LES antibodies and that their inhibition by the antibodies produces the characteristic neuromuscular defect in this disease.

Lambert–Eaton syndrome (LES) is a presynaptic disorder characterized by impaired quantal release of acetylcholine (ACh) at the neuromuscular junction (Elmqvist & Lambert, 1968). LES is often associated with small-cell lung cancer (SCLC; O'Neill, Murray & Newsom-Davis, 1988), a neuroendocrine tumour that expresses neuronal voltage-dependent Ca^{2+} channels (VDCCs; Viglione, O'Shaughnessy & Kim, 1995). Current evidence supports the idea that the syndrome, which has an autoimmune origin, is a result of the inhibition of presynaptic VDCCs by the channel-specific autoantibodies (reviewed in Vincent, Lang & Newsom-Davis, 1989; Wray, 1990). Although the precise immunogen(s) has not yet been determined, an autoimmune response against tumour antigen(s) is thought to trigger the production of pathogenic autoantibodies (Roberts, Perera, Lang, Vincent & Newsom-Davis, 1985; Viglione *et al.* 1995).

Morphological and electrophysiological studies collectively support the view that the LES antibodies downregulate the presynaptic Ca^{2+} channels mediating exocytotic release of ACh from the motor nerve terminal. In freeze-fracture electron micrographs of presynaptic membranes of the neuromuscular junctions of LES patients (Fukunaga, Engel, Osame & Lambert, 1982), active zone particles, hypothesized to be VDCCs, were spatially disorganized and fewer in number. Exposure of cultured neuronal and endocrine cells to IgG from LES patients causes a pronounced reduction in whole-cell currents carried by VDCCs (Kim & Neher, 1988; Peers, Lang, Newsom-Davis & Wray, 1990; Grassi, Magnelli, Carabelli, Sher & Carbone, 1994; Garcia & Beam, 1996; Meriney, Hulsizer, Lennon & Grinnell, 1996). Individual channel activation kinetics and conductance were unchanged, however, indicating that the pathogenic IgG acts to downregulate the number of functional channels (Kim & Neher, 1988; Grassi *et al.* 1994). LES antibodies also reduce depolarization-induced uptake of $^{45}Ca^{2+}$ by SCLC cell lines (Roberts *et al.* 1985; De Aizpurua, Lambert, Griesman, Olivera & Lennon, 1988). As with antibodies against acetylcholine receptors (AChRs) in myasthenia gravis (MG), antigenic modulation and cross-linking of the channels are the events associated with pathogenic action of LES IgG (Peers, Johnston, Lang & Wray, 1993).

Serological studies have also revealed the pathogenic interaction of LES autoantibodies with specific VDCCs. LES IgG can immunoprecipitate N-type VDCCs labelled with [^{125}I]ω-conotoxin GVIA from chick and rat brain synaptosomes (Martin-Moutot, Lang, Newsom-Davis & Seager, 1995), human neuroblastoma cells (Sher *et al.* 1989) and SCLC cells (De Aizpurua *et al.* 1988). More recent work, however, indicates that 95% of LES patients possess antibodies which immunoprecipitate P and/or Q-type VDCCs labelled with [^{125}I]ω-conotoxin MVIIC (Lennon *et al.* 1995).

Despite overwhelming evidence for an autoimmune pathogenesis, no animal model of LES, such as that so well established for MG (Lindstrom, 1979), has been fully developed and characterized. In human myasthenia gravis, a postjunctional disorder of the skeletal neuromuscular junction, AChR proteins isolated from postsynaptic membranes of *Torpedo* electric organ have served as a highly valuable antigen in inducing the anti-AChR antibody production. In an attempt to produce a similar animal model by active immunization, we have recently immunized mice and rats with *Narke japonica* synaptosomes containing cholinergic presynaptic nerve terminals (Kim *et al.* 1998). Consistent with the presynaptic impairment characterizing LES, neuromuscular junctions in these immunized animals exhibit a reduction in the quantal content of nerve-evoked end-plate potentials (EPPs). In a previous study (Chapman, Rabinowitz, Korczyn & Michaelson, 1990), animals injected with similar cholinergic synaptosomes from *Torpedo* were also found to develop electromyographic signs of the syndrome. These studies, however, were not able to address which components of the synaptosome are involved in the autoimmune disease process. The absence of such information thus provides further impetus to identify and characterize the putative Ca^{2+} channel antigens contained in these electric ray synaptosomes.

The aim of the present study is threefold. First is to characterize the types of Ca^{2+} channels present in the electric organ synaptosomes of the Japanese electric ray, *Narke japonica*. Second is to provide direct evidence that LES autoantibodies reduce the depolarization-induced ACh release from these synaptosomes. Third, we explored which types of Ca^{2+} channels are impaired by LES autoantibodies, thus suggesting the type(s) implicated in the disease process.

Synaptosomes from electric organs used in the present work consist of pure cholinergic nerve terminals and are free from postsynaptic membranes. The ACh release characteristics in this preparation are similar to those at the neuromuscular junction (Dunant & Muller, 1986). Thus the results obtained using the synaptosomes are likely to be relevant to the neuromuscular junction. Furthermore, our ability to assess the reduction in I_{Ca} by LES antibodies in bovine adrenal chromaffin cells and to correlate this reduction with the inhibition of synaptosomal ACh release provides compelling evidence that LES antibodies interact with Ca^{2+} channels functionally coupled to the transmitter release. In addition to their value in the induction of experimental autoimmune Lambert–Eaton syndrome, cholinergic nerve terminals of electric organ appear to be an ideal system with which to assess directly the action of LES IgG on cholinergic transmitter release.

METHODS

Chemicals

Horseradish peroxidase, acetylcholine chloride, luminol and 3-aminobenzoic acid ethyl ester methanesulphonate (tricaine) were obtained from Sigma (USA). Choline oxidase was purchased from Funakoshi (Tokyo, Japan) and peroxidase (from *Arthromyces ramous*) from Nakalai Tesque (Kyoto, Japan). Ionomycin was from Calbiochem (USA). Synthetic ω-conotoxin GVIA (ω-CgTX GVIA), ω-conotoxin MVIIC (ω-CTX MVIIC), ω-agatoxin IVA (ω-AgTX IVA) and calciseptine were obtained from Peptide Institute (Osaka, Japan).

Preparation of electric organ synaptosomes

Preparation of synaptosomes from electric organ was carried out at 4 °C using sucrose density gradient centrifugation as described previously (O'Hori *et al.* 1993). Japanese electric rays, *Narke japonica*, were captured off the coast of Nagasaki and Fukuoka Prefectures, Japan, and were kept alive in artificial sea water (Yashima, Osaka, Japan) until use. After anaesthesia with 0·03% tricaine, fish (typically 5) were dissected to isolate electric organs. Finely chopped organs were suspended in modified physiological medium (MPM) containing the following (mM): 285·1 NaCl, 3 KCl, 1·8 $MgCl_2$, 100 sucrose, 5·5 glucose, 300 urea and 40 Na-Hepes (pH 7·4). The suspension was stirred for 30 min and successively passed through nylon mesh with square openings of 1000, 500, 200 and 50 μm. The filtrate was centrifuged at 6000 *g* for 20 min. The

pellet was resuspended in physiological medium and layered on top of a stepwise sucrose density gradient. The constituents of each layer from the top were as follows: MPM containing 0·1 M urea and 0·3 M sucrose instead of 0·3 M urea and 0·1 M sucrose; and MPM containing 0·5 M sucrose instead of 0·3 M urea and 0·1 M sucrose. Centrifugation was performed at 63 000 *g* for 40 min. The band at the interface of 0·1 M urea plus 0·3 M sucrose and 0·5 M sucrose was collected and centrifuged at 95 500 *g* for 60 min. The pellet was resuspended in MPM and used for experiments within 12 h.

Continuous measurement of acetylcholine release

ACh release from synaptosomes evoked by depolarization with high concentrations of KCl was measured continuously at physiological pH 7·4 using the chemiluminescence method, according to Schweitzer's modification (Schweitzer, 1987) of the original method by Israël & Lesbats (1981). We previously confirmed that under our experimental conditions described below, light output was linear with respect to the amount of ACh over the range investigated (O'Hori *et al.* 1993).

The procedure for the measurement of ACh released from synaptosomes untreated with IgGs was as follows: a 30 μl suspension of synaptosomes in MPM (about 1 mg ml^{-1} protein) was mixed with an MPM solution (120 μl) containing the chemiluminescence agents (choline oxidase, peroxidase, luminol) and 4·25 mM $CaCl_2$, with or without the addition of Ca^{2+} channel blockers. After incubation in a cuvette of a photometer (Lumicounter 1000, Niti-on, Funabashi, Japan) at 25 °C for 20 min, the depolarization stimulus was applied by injection of 100 μl isotonic high $[K^+]_o$ solution (mM): 37·5 NaCl, 245·5 KCl, 1·8 $MgCl_2$, 5·5 glucose, 3·4 $CaCl_2$, 50 urea, 400 sucrose and 40 Na-Hepes, pH 7·4, with a microsyringe through a rubber septum. The photometer is equipped with a device that rotates the cuvette horizontally and reciprocally, which ensures a complete mixing of the contents in the cuvette. The final composition of the contents (250 μl) in the cuvette was about (mM): 0·06 luminol, 183 NaCl, 100 KCl, 1·8 $MgCl_2$, 3·4 $CaCl_2$, 5·5 glucose, 50 urea, 400 sucrose and 40 Na-Hepes (pH 7·4); 120 μg ml^{-1} protein, 20 U ml^{-1} choline oxidase and 20 μg ml^{-1} peroxidase.

The procedure for the measurement of ACh released from IgG-treated synaptosomes was as follows: a 30 μl suspension of synaptosomes in MPM (about 1·2 mg ml^{-1} protein) was mixed with an MPM solution (120 μl) containing the chemiluminescence agents (choline oxidase, peroxidase, luminol) and 4·25 mM $CaCl_2$, with the addition of LES IgG or serum. After incubation for 70 min at 25 °C, the suspension was mixed with 30 μl of MPM or MPM containing Ca^{2+} channel blockers and incubated for another 20 min. Then, to induce depolarization, 120 μl of isotonic high $[K^+]_o$ solution of the same composition as above was injected into the sample. The final composition of the contents (300 μl) was the same as above except for the presence of IgG or serum. The amount of ACh released was determined from the area under the peak of the chemiluminescence curve, as compared with that of standard ACh added to the sample at the end of measurement.

Preparation of LES serum and IgG

Three LES patients, whose serological samples were utilized in the present study, were clinically and electromyographically diagnosed as having LES. Patients 1 and 2 (from the University of Virginia Hospital) had associated small-cell lung cancer and their samples were identified as having antibodies capable of inhibiting I_{Ca} in patch-clamp studies (see Results). Patient 3 (from Toneyama Hospital, Osaka, Japan) also manifested SCLC which was confirmed at autopsy; this patient's serum was not assayed for its effect on I_{Ca}.

LES plasma, collected during plasma exchange therapy from patients 1 and 2, was converted to serum by Ca^{2+}-induced precipitation of clotting factors. Immunoglobulin G was isolated from plasma using the Rivanol precipitation method. Blood was withdrawn by venipuncture from patient 3 and allowed to clot at room temperature (22–25 °C) and then serum was obtained by centrifugation at 700 *g* for 10 min. The serum or IgG was then dialysed for 48 h against the culture medium with a dialysate change at 24 h. Concentration of IgG was determined by rate flow nephelometry in the Clinical Immunology Laboratory. Control plasma (from healthy individuals) was obtained from the University of Virginia blood bank, and control serum or IgG prepared as described above.

All human serological samples and the synaptosomal preparations used in this study were obtained in conformity with the guidelines established by the institutional Human and Animal Investigation Committees at the University of Tokyo and University of Virginia.

Patch-clamp study of the effects of LES IgG on whole-cell calcium currents

We used standard patch-clamp techniques to record voltage-dependent Ca^{2+} and Na^+ currents (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) from bovine adrenal chromaffin cells using a List EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Preparation of the cultured cells and patch-clamp recording procedures are the same as described elsewhere (Kim & Neher, 1988). The following solutions were used for all patch-clamp recordings of I_{Ca}. The internal or pipette solution contained (mM): 1 $CaCl_2$, 2 $MgCl_2$, 120 CsCl, 11 Na-EGTA, 20 TEACl, 10 Na-Hepes (pH 7·2). For most patch-clamp recordings, the bath contained (mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 5 glucose, 10 Na-Hepes (pH 7·2). A 20 mV test potential applied from the holding potential at a rate of 5 Hz was used to monitor the establishment of the gigaohm seal. After seal formation, values for membrane capacitance (C_m) and access resistance (R_s) were obtained by cancellation of the transient current with the EPC-7. Measurements were initiated 1 min after achieving the whole-cell configuration and all experiments were performed at room temperature (22–25 °C).

Data collection and statistics

In most of the synaptosome studies, chemiluminescence measurements of ACh release were repeated five times for each preparation under a particular test condition. In each patch-clamp experiment, I_{Ca} was sampled from eight to twelve bovine adrenal chromaffin cells subjected to incubation with control or LES IgG/serum. All data were pooled for each experimental condition and presented as means ± S.E.M. of all measurements. Statistical significance of the difference between the two means was tested using Student's *t* test. Unless otherwise noted, *P* values less than 0·05 were considered to be statistically significant.

RESULTS

Pharmacological characterization of VDCCs in electric organ synaptosomes

Utilizing the sensitivity to Ca^{2+} channel blockers specific to subtypes of VDCCs, we characterized the VDCCs of electric organ synaptosomes from the Japanese electric ray, *Narke japonica*, a member of Torpedinidae family. When

synaptosomes were depolarized, ACh was released in a manner dependent on the strength of depolarization and on the extracellular Ca^{2+} concentration. Throughout the present study, depolarization was induced by increasing extracellular K^+ concentration from 3 to 100 mM in the presence of 3·4 mM extracellular Ca^{2+}.

The presence of N-type VDCCs sensitive to ω-CgTX GVIA in the electric organ synaptosomes has been established (Ahmad & Miljanich, 1988; Fariñas, Solsona & Marsal, 1992; O'Hori *et al.* 1993). The ω-CgTX GVIA-sensitive VDCC pharmacologically identified in these studies appears to correspond to the expression of a cDNA clone, doe-4, for the α_1-subunit of a putative VDCC from the electric lobe (Horne, Ellinor, Inman, Zhou, Tsien & Schwarz, 1993). The sequence of doe-4 has about a 70% homology with that of the mammalian B subclass which corresponds to the α_1-subunit of the N-type VDCC. This provides a rationale for the use of the same criteria applied for mammalian preparations in classifying the Ca^{2+} channel types in electric fish.

The dose–inhibition curve obtained after exposing the synaptosomes to varying concentrations of ω-CgTX GVIA is illustrated in Fig. 1 *A*. When fitted by a Michaelis–Menten-type equation, the curve gave rise to a half-inhibition concentration (IC_{50}) of 7·5 μM and maximum inhibition (I_{max}) of 47%. In electric organ synaptosomes, the dissociation constant (K_d) of ω-CgTX GVIA with N-type VDCCs is several micromolars (O'Hori *et al.* 1993), similar to the observed IC_{50} in this study. More than 50% of ACh release was not blocked by ω-CgTX GVIA, suggesting that the remaining release may be due to Ca^{2+} influx through ω-CgTX GVIA-insensitive VDCCs.

To determine the expression of P-type VDCCs in the *Narke japonica* nerve terminals, we examined the effect of ω-AgTX IVA, a P-type VDCC-specific antagonist (Mintz, Adams & Bean, 1992). Shown in Fig. 1 *B* is the dose–inhibition relationship with ω-AgTX IVA, from which IC_{50} at 2·6 μM and I_{max} of 43% were derived. ω-AgTX IVA, at concentrations higher than 200 nM has

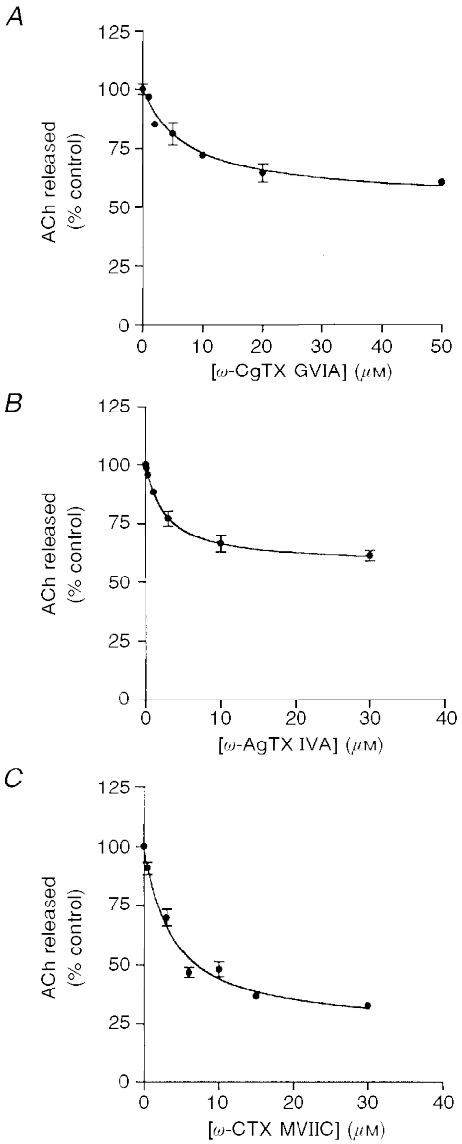

Figure 1. Inhibition of high $[K^+]_o$-induced ACh release in electric organ synaptosomes by Ca^{2+} channel antagonists

ω-CgTX GVIA (*A*), ω-AgTX IVA (*B*) and ω-CTX MVIIC (*C*) block transmitter release from the cholinergic nerve terminals in a dose-dependent manner. Each value is the mean ± S.E.M. (bars) from at least 3 independent determinations. The continuous line is a curve best fitted using a Michaelis–Menten type equation with $IC_{50} = 7·5$ μM, $I_{max} = 47·0$% for *A*; $IC_{50} = 2·6$ μM, $I_{max} = 42·5$% for *B*; and $IC_{50} = 3·8$ μM, $I_{max} = 77·1$% for *C*.

been found to partially block Q-type VDCCs (Sather, Tanabe, Zhang, Mori, Adams & Tsien, 1993). The difference between Q- and P-types of VDCCs seems to be the concentrations at which ω-AgTX IVA block these channels. Since the affinity of ω-AgTX IVA in electric organ synaptosomes is much lower than that in mammalian preparations, it is difficult to determine if the VDCC in electric organ synaptosomes inhibited by the micromolar concentrations of ω-AgTX IVA is of P- or Q-type. Therefore, we have designated ω-AgTX IVA-sensitive VDCCs of the electric organ synaptosomes as 'P/Q-type' VDCCs.

Next, we examined whether or not the inhibitory effect of ω-AgTX IVA is independent of that of ω-CgTX GVIA. When ω-AgTX IVA at 30 μM was applied together with a maximal concentration of ω-CgTX GVIA (50 μM), the K^+-evoked ACh release was inhibited by 69·9 ± 2·2 % (mean ± S.E.M., $n = 3$, three different experimental runs using a single preparation) while 50 μM ω-CgTX GVIA alone exerted 47·0 ± 4·2 % inhibition ($n = 5$). The additional inhibition of ω-AgTX IVA over ω-CgTX GVIA indicates a different site of action of ω-AgTX IVA from that of the N-type VDCC (Fig. 2).

Although ω-CTX MVIIC is usually assumed to be a P-type-specific VDCC antagonist, it inhibits not only P- but also N-type VDCCs at high concentrations (Turner, Lampe & Dunlap, 1995). Accordingly, ω-CTX MVIIC at its maximum dose is expected to produce a greater effect than the maximum inhibition by ω-CgTX GVIA or ω-AgTX IVA. Figure 1*C* shows the dose-dependent blockade of the ACh release by ω-CTX MVIIC. When applied at the maximum dose, the toxin blocked ACh release by 71 %, a value exceeding the maximum effect of either ω-CgTX GVIA or ω-AgTX IVA. Addition of either ω-CgTX GVIA or ω-AgTX IVA to synaptosomes pretreated with ω-CTX MVIIC yielded no further reduction (Fig. 2). These results strongly suggest that ω-CTX MVIIC inhibits both N- and P-type VDCCs of the electric organ synaptosomes. Therefore, ω-AgTX IVA, not ω-CTX MVIIC, was used in the following experiments to discriminate P/Q-type and N-type VDCCs.

Although L-type VDCC is also involved in cholinergic transmitter release from electric organ synaptosomes, its contribution is smaller than those of N- or P/Q-type VDCC (Tokumaru, Satoh, Hirashima, Shojaku, Yamamoto & Kirino, 1997). Calciseptine, a potent inhibitor of L-type VDCCs, maximally depressed the K^+-evoked ACh release by only about 20 %. This is in accord with our previous data (Tokumaru *et al.* 1995) demonstrating inhibition by a monoclonal antibody against the $\alpha_2\delta$-subunit of L-type VDCC and provides evidence for involvement of the L-type VDCCs in ACh release. Calciseptine caused additional depression of ACh release from synaptosomes treated with ω-AgTX IVA and ω-CgTX GVIA (Fig. 2). Therefore, the three toxins used in this study inhibit ACh release independently, acting on different subtypes of VDCCs.

Effects of LES IgG on ACh release

Prior to high K^+ stimulation, synaptosomes were pre-incubated at 25 °C for varying time periods with serum from LES patient 1 or with control (disease-free) human serum. Upon incubation with control serum (0·5 mg ml^{-1} IgG) for 90 min, the synaptosomes released a total ACh of 71·3 ± 5·1 pmol ($n = 3$; Fig. 3*A*). The equivalent amount of ACh released in preparations incubated with LES serum (patient 1, 0·5 mg ml^{-1} IgG) was distinctively lower: 34·0 ± 4·3 pmol ($n = 3$, $P < 0·01$). The time course of the inhibitory action of LES serum on ACh release is shown in Fig. 3*B*. After 75 min of incubation, we observed maximal inhibition of about 50 %, as compared with the release from synaptosomes incubated with serum from healthy individuals.

In a different synaptosomal preparation incubated for 90 min with the same LES serum, we also observed a decrease in the amount of ACh released to 53·8 ± 5·1 pmol ($n = 5$, 45 % of control, $P < 0·0001$) from control value of 119·2 ± 3·5 pmol (incubated with control serum for 90 min, $n = 5$).

We further confirmed the antibody-induced inhibition of cholinergic transmitter release using serum from two additional LES patients. After 90 min of incubation, IgG

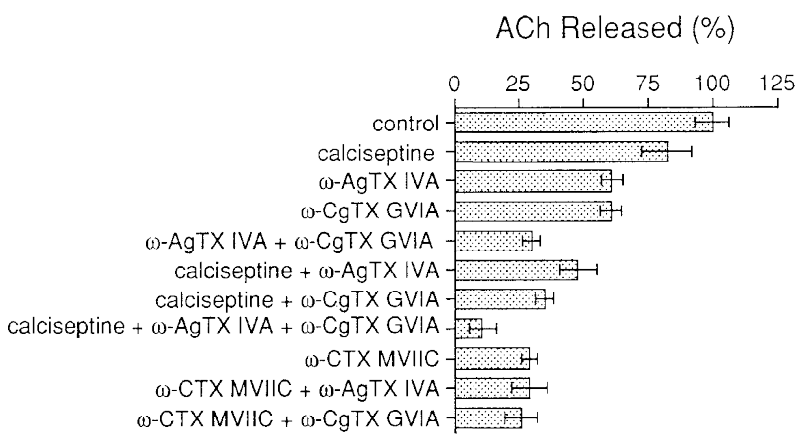

Figure 2. Inhibitory effects of various combinations of Ca^{2+} channel blockers on evoked ACh release from synaptosomes

Concentration of antagonists was 30 μM for calciseptine, ω-AgTX IVA and ω-CTX MVIIC, and 50 μM for ω-CgTX GVIA. Each value represents the mean ± S.E.M. (bars) from at least 3 determinations.

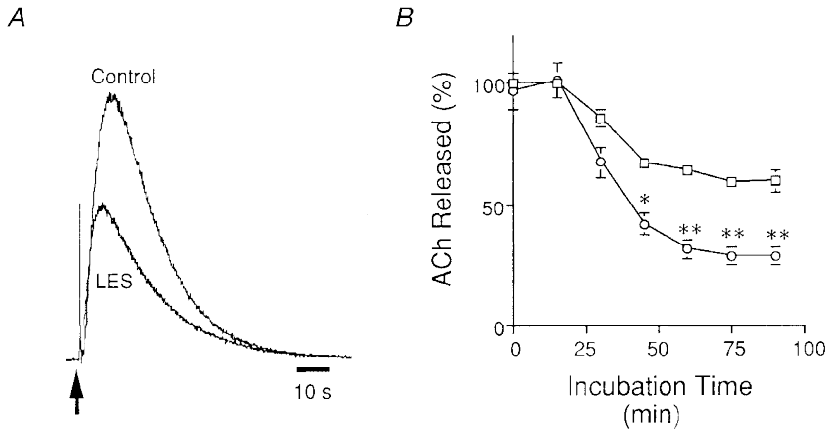

Figure 3. LES IgG diminishes evoked ACh release from electric organ synaptosomes

Synaptosomes were incubated with LES serum (0·5 mg ml^{-1} IgG) at 25 °C and then depolarized by injection of the high $[K^+]_o$ solution to increase K^+ concentration in the medium from 3 to 100 mM. The experiments were carried out using a single preparation of synaptosomes. The ACh released was measured by the chemiluminescence method. See Methods for details. *A*, chemiluminescence output from electric organ synaptosomes incubated for 90 min with sera from LES patient 1 or control subject. In this particular example, ACh released was 73·6 pmol (Control) and 37·6 pmol (LES). Arrow indicates the time high $[K^+]_o$ stimulation was applied. *B*, the time course of the inhibitory action of LES IgG on ACh release from electric organ synaptosomes. The effects of LES (○) and control (□) sera (0·5 mg ml^{-1} IgG) on ACh release were plotted as a function of incubation time. Notice that inhibition reaches maximum after about 60 min of incubation. The decrease of ACh release in control (and in LES in part) is due to incubation of the synaptosomes at a high temperature (22–25 °C). Each value is the mean ± S.E.M. (bars) from 5 independent determinations. * $P < 0·05$ and ** $P < 0·01$, by Student's t test compared with control at the same incubation time.

(1 mg ml^{-1}) from patient 2 markedly diminished ACh release to 46·4 ± 3·7 pmol ($n = 5$, 39% of control, $P < 0·0001$). Likewise, ACh liberated upon exposure to serum from patient 3 with the same IgG concentration was significantly smaller, 49·5 ± 2·9 pmol ($n = 5$; 42% of control; $P < 0·0001$).

Effects of LES IgG on ionomycin-induced release of ACh

The reduction in cholinergic transmitter release by LES IgG may be caused by altered intracellular Ca^{2+} homeostasis or exocytotic secretion. In view of this possibility, we determined whether the pathological antibodies act on steps beyond Ca^{2+} influx through VDCCs. To this end, we used the ionophoric polyether antibiotic, ionomycin, which selectively increases membrane permeability to Ca^{2+} ions, facilitating Ca^{2+} influx independent of VDCCs. Synaptosomes incubated with serum (0·5 mg ml^{-1} IgG) from LES patient 1 or a healthy individual were stimulated with 1 mM ionomycin. As shown in Fig. 4, there was little difference in the release profiles between the two, signifying that LES IgG failed to interfere with the ionomycin-induced release of ACh. These findings thus indicate that the cholinergic

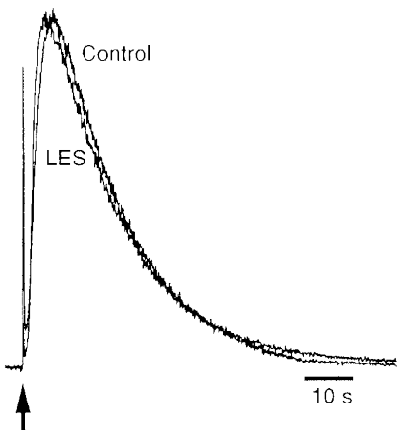

Figure 4. LES IgG has no effect on ionomycin-evoked transmitter release

Synaptosomes were incubated for 90 min with serum (0·5 mg ml^{-1} IgG) from LES patient 1 or a control healthy individual, followed by stimulation with 1 mM ionomycin. Little difference in the amount of released ACh was observed between LES and control serum-treated terminals. Arrow indicates the time ionomycin was applied.

terminals treated with LES IgG are capable of producing normal secretory activity when sufficient Ca^{2+} ions become available intracellularly. This in turn supports the notion that LES IgG impairs neither intracellular Ca^{2+} metabolism nor the ACh secretory machinery.

LES IgG inhibits I_{Ca} in chromaffin cells

With the purpose of correlating the synaptosomal effects with the functional inhibition of VDCCs, we explored whether antibodies from patients 1 and 2 were capable of inhibiting I_{Ca} in bovine adrenal chromaffin cells. Bovine adrenal chromaffin cells are a well-studied neuroendocrine system known to undergo calcium-dependent exocytosis (Kim & Neher, 1988). These cells express L-, N-, and P-type VDCCs (Albillos, Garcia, Olivera & Gandia, 1996), which are sensitive to antagonists similar to those interacting with electric organ VDCCs.

We examined I_{Ca} from chromaffin cells after they had been exposed to IgG (1 mg ml^{-1}) from LES patient 2 for 24 h. The peak I_{Ca} elicited at +10 mV declined markedly to 13·3 pA pF^{-1} ($n = 24$ cells) compared with 24·5 pA pF^{-1} ($n = 23$) measured in cells incubated in control IgG, a reduction of 46% (Fig. 5 *A*). The current–voltage relationships are unchanged by the presence of the pathogenic antibodies, only the amplitude of the current is altered (Fig. 5 *B*). Serum from patient 1 yielded similar results (Fig. 5 *C*).

Type of VDCCs downregulated by LES antibodies

These experiments are based on the premise that the receptors for the toxins are integral parts of the Ca^{2+}

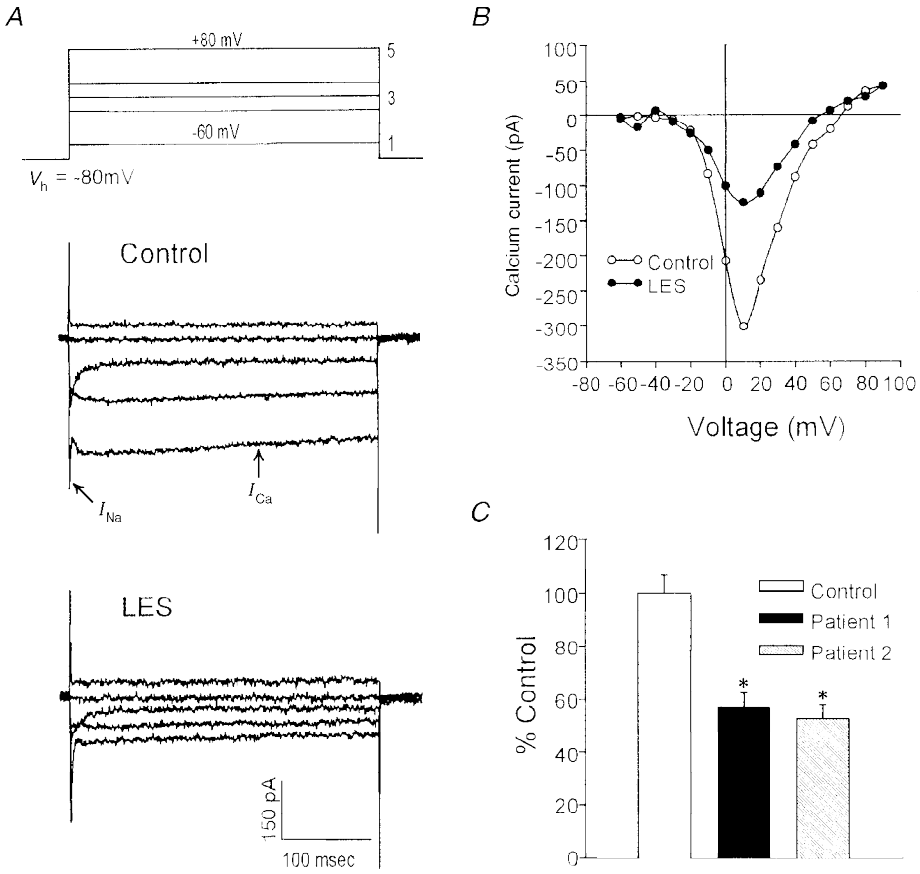

Figure 5. Patch-clamp recordings of whole-cell I_{Ca} in bovine adrenal chromaffin cells

A, shown are representative Ca^{2+} currents from bovine adrenal chromaffin cells incubated with control and LES (patient 2) IgG (2 mg ml^{-1}) for 24 h. The 5 depolarizing voltage steps applied are shown at the top of the panel: from the holding potential of −80 mV to −60, −10, +10, +30 and +80 mV. The maximal current was elicited with step 3 to +10 mV. The scale bar is for both control and LES tracings. *B*, current–voltage (*I–V*) relationship for the cells presented in *A*. Notice that the maximal current occurs at +10 mV for both cells and that the general relationship is unaltered by the presence of the LES antibodies. *C*, summary data for peak I_{Ca} in bovine adrenal cells depolarized to +10 mV. Cells were exposed for 24 h to serum from LES patient 1 (1 mg ml^{-1} IgG), IgG from LES patient 2 (2–3 mg ml^{-1} IgG), or equivalent control serum/IgG. The data show means ± S.E.M. ($n = 31$ cells for Control; $n = 16$ for patient 1, and $n = 24$ for patient 2) and have been normalized such that the control mean is 100%. * $P < 0{\cdot}001$ by Student's *t* test compared with control.

channel complex. Therefore, the efficacy of a given toxin specific to a VDCC type, applied subsequent to LES IgG treatment, will be compromised if the pathogenic antibodies downregulate that particular channel type. This would allow identification of the type of VDCC which must have been inhibited by the antibodies and thus causing a reduction in the release of ACh from the synaptosomes.

After incubation with LES serum for 70 min, synaptosomes were exposed to one of the three Ca^{2+} channel blockers for another 20 min. Then, its ability to affect the ACh release was assessed. We first tested the effect of ω-CgTX GVIA (50 μM) following the preincubation with LES serum (0·5 mg ml^{-1} IgG; Fig. 6 *A*). As described previously, LES serum from patient 1 reduced the ACh release to 45·1 $\pm$ 4·3 % of control (0·5 mg ml^{-1} normal IgG). When these pretreated synaptosomes were incubated with 50 μM ω-CgTX GVIA, the ACh release was 11·9 $\pm$ 2·6 % ($n = 5$) relative to the control serum. Thus, the toxin produced an additional 33 % decrease beyond that achieved with LES serum ($P < 0·001$). Therefore, ω-CgTX GVIA-sensitive component of ACh release was little affected by LES IgG, indicating that the N-type VDCCs were not downregulated by the autoantibodies.

Next, to examine whether LES IgG inhibits ω-AgTX IVA-sensitive P/Q-type VDCCs, we tested the efficacy of ω-AgTX IVA with the same protocol. When synaptosomes were pretreated with LES serum, ω-AgTX IVA (30 μM) was essentially ineffective in inhibiting ACh release (Fig. 6 *B*). In control serum-treated synaptosomes, ω-AgTX IVA (30 μM) reduced ACh release by 35·0 $\pm$ 2·7 %. The amount of ACh released in the presence of LES serum and ω-AgTX IVA was 40·4 $\pm$ 3·4 % ($n = 5$) of the control, which is only an additional 4·7 % inhibition and statistically not significant. Thus, autoantibodies from LES patient 1 nearly abolished the efficacy of ω-AgTX IVA, suggesting that ω-AgTX IVA-sensitive VDCCs are indeed downregulated by LES IgG.

Finally, we tested the action of calciseptine on synaptosomes pretreated with LES antibodies. As shown in Fig. 6 *C*, calciseptine (10 μM) applied under this condition diminished ACh release by an additional 13·5 $\pm$ 3·4 % (ACh released was 37·7 $\pm$ 4·1 pmol, $n = 5$). This extent of inhibition is almost the same as that obtained with application of calciseptine (10 μM) to the control serum-incubated preparations (14·7 %). Thus the calciseptine-sensitive component of ACh release was negligibly affected by the pathological antibodies. Effects of the Ca^{2+} channel antagonists on the cholinergic transmitter release from synaptosomes preincubated with LES serum are summarized in Table 1.

Application of IgG or serum from two additional patients to synaptosomes confirmed the selective loss of efficacy of ω-AgTX IVA. In the two LES preparations, ω-CgTX GVIA was effective with an additional blockage of ACh release by 35–38 %, whereas ω-AgTX IVA blocked the release by only 3·3–3·8 %. Thus, we conclude that pathogenic LES antibodies induce the downregulation of P/Q-type VDCCs of electric organ synaptosomes.

DISCUSSION

The electric organ synaptosomes from the Japanese electric ray, *Narke japonica*, described herein, have recently been used as an effective autoantigen in the induction of experimental autoimmune Lambert–Eaton syndrome (Kim *et al.* 1998). Mice and rats immunized with these cholinergic

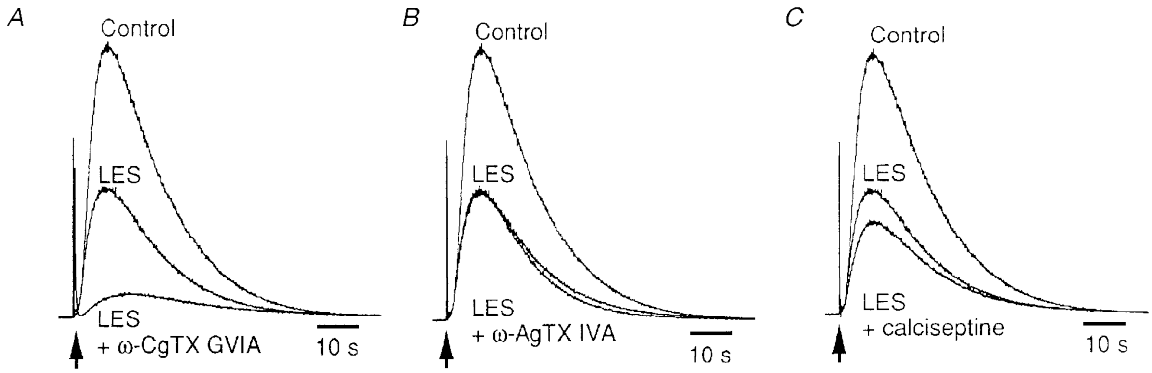

Figure 6. ACh release from synaptosomes preincubated with LES IgG is less sensitive to ω-AgTX IVA than to ω-CgTX GVIA or calciseptine

Traces of chemiluminescence output are shown. They are outputs from electric organ synaptosomes incubated with control serum (Control), LES serum (LES), and LES serum plus one of the following Ca^{2+} channel antagonists: ω-CgTX GVIA (*A*), ω-AgTX IVA (*B*) and calciseptine (*C*). The amount of released ACh obtained from Control and LES traces is 120·5 and 54·1 pmol, respectively. When applied following treatment with LES serum, ω-CgTX GVIA, ω-AgTX IVA and calciseptine further reduced ACh secretion to 10·3, 48·6 and 38·1 pmol, respectively (as depicted in the bottom traces of *A*, *B*, and *C*). Arrows indicate the time when high K^+ stimulation was applied. The same control and LES sera (0·5 mg ml^{-1} IgG) from patient 1 were used throughout this study.

Table 1. Effects of Ca^{2+} channel antagonists on ACh release from synaptosomes preincubated with LES autoantibodies

Pretreatment	ACh release (pmol)			
	No antagonist	ω-CgTX GVIA	ω-AgTX IVA	Calciseptine
Control	119·2 ± 3·5 (100 ± 2·9 %)	67·2 ± 5·0** (56·4 ± 4·2 %)	77·5 ± 4·1** (65·0 ± 2·7 %)	101·7 ± 5·2* (85·3 ± 4·4 %)
LES	53·8 ± 5·1 (45·1 ± 4·3 %)	14·2 ± 3·1 † (11·9 ± 2·6 %)	48·1 ± 4·0 (40·4 ± 3·4 %)	37·7 ± 4·1 ‡ (31·6 ± 3·4 %)

** $P < 0·0001$ compared with the value found in control cells with no antagonist. * $P < 0·05$ compared with the value found in control cells with no antagonist. † $P < 0·001$ compared with the value found in LES cells with no antagonist. ‡ $P < 0·05$ compared with the value found in LES cells with no antagonist. Synaptosomes (30 μg protein) were preincubated with control and LES (patient 1) serum samples (0·5 mg ml^{-1} IgG), followed by incubation with Ca^{2+} channel antagonists. The amount of released ACh is averaged (means ± S.E.M.) from at least 4 determinations. The percentage values, relative to the control obtained from control cells with no antagonists, are shown in parentheses.

nerve terminals exhibit a prejunctional defect of neuromuscular transmission characterized by a reduction in end-plate potential quantal content. However, the type(s) of synaptosomal VDCCs acting as the autoantigen has not been identified. In the present work, we have pharmacologically classified the VDCCs of the cholinergic terminal synaptosomes, revealing the existence of at least three types of Ca^{2+} channels: ω-CgTX-sensitive N-type, ω-AgTX IVA-sensitive P/Q-type and calciseptine-sensitive L-type VDCCs. To determine the reactivity of LES IgG with the putative Ca^{2+} channel antigens, we have also probed the functional impairment of the cholinergic transmitter release caused by the autoantibodies. These experiments prove that the pathogenic LES IgG predominantly downregulates ω-AgTX IVA-sensitive P/Q-type Ca^{2+} channels. It is noteworthy that this Ca^{2+} channel type is coupled with ACh exocytotic secretion in electric organ synapses, as is the case with the motor nerve terminals of the mammalian neuromuscular junction (Protti & Uchitel, 1993; Protti, Reisin, Mackinley & Uchitel, 1996). These findings provide the rationale for the use of electric organ synaptosomes as an immunogen to develop an autoimmune animal model of LES.

Cholinergic nerve terminals of *Narke japonica* electric organ appear to be an excellent system in which to study the specificity of LES IgG for multiple presynaptic Ca^{2+} channels that mediate neurotransmitter release process. The present results demonstrate that ACh release in electric organ synaptosomes is inhibited by acute application of serum or IgG from LES patients. In synaptosomes incubated with LES IgG, ionomycin-induced ACh release was not changed from the control, suggesting that the reduction in ACh release was not a result of an effect downstream of Ca^{2+} influx through VDCCs. Thus, the inhibition caused by the pathogenic autoantibodies is attributed to alteration in the activity of VDCCs at the presynaptic nerve terminal. This notion is confirmed by the experiments showing that antibodies from two of the LES patients, which were effective in reducing ACh release from the synaptosomes, also inhibit I_{Ca} in bovine adrenal chromaffin cells.

Significant variations in the time course of the effect of LES antibodies have been reported. A reduction in $^{45}Ca^{2+}$ uptake or voltage-dependent Ca^{2+} currents in SCLC cell lines was only apparent after 24 h incubation with IgG and was maximal after 1 to 4 days (De Aizpurua *et al.* 1988; Viglione *et al.* 1995). This is in contrast with the report that half-maximal inhibition of Ca^{2+} influx in SCLC cells (MB) derived from an LES patient occurred following a 90 min incubation (Johnston, Lang, Leys & Newsom-Davis, 1994). The early inhibition of whole-cell I_{Ca} in bovine adrenal chromaffin cells and an SCLC cell line occurred after 0·5 and 2 h exposures to LES IgG, respectively, (Kim & Neher, 1988; Meriney *et al.* 1996). In both rat and chick synaptosomes, an incubation period lasting 1 h was sufficient to allow LES antibody binding (Martin-Moutot *et al.* 1995). The time course of the effect of LES antibodies observed from electric organ synaptosomes (half-maximal inhibition occurred in about 30 min, Fig. 3 *B*) was relatively fast and similar to that observed in some of these preparations. Among the factors responsible for the varying time courses is the different 'potency' or concentration of pathogenic antibodies used in these studies. Additional plausible explanation is that frequency of autoantibody cross-linking of VDCCs (Peers *et al.* 1993), a phenomenon required for antigenic modulation, may vary depending upon density of the putative Ca^{2+} channels in a given preparation. The cross-linkage may be delayed, for example, with low density of VDCCs interacting with LES IgG, resulting in a prolonged time course of the IgG-induced downregulation.

As isolation of large quantities of motor nerve terminals from skeletal neuromuscular junctions is impractical, synaptosomes from mammalian cortical synaptosomes have

been used as a model system to study the synaptic inhibitory action of LES antibodies. In rat forebrain synaptosomes, ω-CgTX GVIA or dihydropyridines do not block ACh release (Hewett & Atchison, 1992) nor inhibit the $^{45}Ca^{2+}$ uptake by K^+-induced depolarization (Pocock, Venema & Adams, 1992; Martin-Moutot *et al.* 1995). Depolarization-induced $^{45}Ca^{2+}$ influx in chick synaptosomes is partially inhibited by ω-CgTX GVIA (Pocock *et al.* 1992; Martin-Moutot *et al.* 1995). It is unclear, however, whether these synaptosomal preparations were previously identified as possessing ω-AgTX IVA-sensitive P/Q-type Ca^{2+} channels, a principal channel subtype downregulated by LES IgG.

Assessment of synaptosomal Ca^{2+} channel function modified by LES IgG has been inconsistent. Hewett & Atchison (1992) found LES serum to reduce depolarization-induced uptake of $^{45}Ca^{2+}$ into rat brain synaptosomes, whereas Martin-Moutot *et al.* (1995) reported no apparent effect of LES sera or IgG on $^{45}Ca^{2+}$ uptake in either rat or chick brain synaptosomes. Unlike our results, inhibition by LES IgG of $^{45}Ca^{2+}$ influx in the brain synaptosomes required the presence of 10% serum (Hewett & Atchison, 1992). Remarkably, LES IgG identified as immunoprecipitating [^{125}I]ω-CgTX GVIA-labelled N-type VDCCs failed to inhibit $^{45}Ca^{2+}$ uptake (Martin-Moutot *et al.* 1995). This raises the possibility that immunoprecipitation assay of ω-CgTX GVIA-sensitive channels may bear no correlation with functional impairment of that particular channel type. Alternatively, the lack of functional effect in synaptosomes may be attributed to the disruption of the cytoskeleton during tissue homogenization (Martin-Moutot *et al.* 1995). This, however, does not explain the positive result obtained with ω-AgTX IVA-sensitive P/Q-type VDCCs, which are both immunoprecipitated and functionally inhibited by LES IgG (Lennon *et al.* 1995; Viglione *et al.* 1995).

The present experiments provide evidence that presynaptic inhibition of ACh release caused by LES IgG, as confirmed with antibodies from three patients, is primarily through ω-AgTX IVA- sensitive P/Q-type VDCCs. It is relevant to recognize functional similarity of these synaptosomal channels and the presynatic Ca^{2+} channels at the neuromuscular junction: both of these channel types are engaged in cholinergic transmitter release at a synaptic site. Downregulation of either ω-CgTX GVIA-sensitive N-type or calciseptine-sensitive L-type Ca^{2+} channels was insignificant. Such Ca^{2+} channel specificity concurs with the two recent studies demonstrating immunoprecipitation of P/Q-type Ca^{2+} channels (Lennon *et al.* 1995) and functional inhibition of SCLC P-type Ca^{2+} channels (Viglione *et al.* 1995) by LES antibodies.

The apparent heterogeneity of Ca^{2+} channel specificities among LES antibodies, however, has also been reported. LES IgG was found to specifically immunoprecipitate ω-CgTX GVIA-sensitive VDCC complex and to increase the rate of ^{125}I-ω-CgTX GVIA receptor internalization and degradation (Lennon & Lambert, 1989; Sher *et al.* 1989). In a variety of neuroendocrine and neuronal cell types, the pathogenic antibodies reportedly inhibit I_{Ca} carried by N-type (Grassi *et al.* 1994), L-type (Peers *et al.* 1990; Grassi *et al.* 1994) and T-type (Grassi *et al.* 1994; Garcia & Beam, 1996) VDCCs. A patch-clamp study of an SCLC cell line treated with LES IgG suggests downregulation of multiple types of Ca^{2+} channels (Meriney *et al.* 1996). In view of the role of P/Q type Ca^{2+} channels in evoked transmitter release at the human neuromuscular junction (Protti *et al.* 1996), it is logical to expect that LES autoantibodies pathologically target this particular channel type. The present finding that LES IgG specifically downregulates P/Q-type VDCCs is consistent with this expectation. The role in pathogenesis of the antibodies immunoprecipitating or inhibiting the function of ω-CgTX GVIA-sensitive VDCCs, a channel type not found at the human and mouse neuromuscular junctions, is yet to be determined. As the autonomic dysfunction is a common clinical symptom of LES (Mamdani, Walsh, Rubino, Brannegan & Hwang, 1985), anti-N-type Ca^{2+} channel antibodies may potentially interfere with transmitter release at the autonomic synapses. In a recent study of passively transferred LES in mice, the function of sympathetic and parasympathetic nerve terminals was assessed, but no evidence supporting such pathophysiology was found (Waterman, Lang & Newsom-Davis, 1997). In the majority of animals tested, the defect of autonomic synaptic transmission did not arise from inhibition of N-type Ca^{2+} channels but due to the inhibition of P/Q-type channels.

It has been proposed that a synaptic protein other than a Ca^{2+} channel is the autoantigen in LES. A vesicle-associated protein, synaptotagmin was asserted as a putative target based on *in vitro* immunoprecipitation studies (Levêque *et al.* 1992). In addition, rats immunized with peptides of synaptotagmin residues (20 to 53 inclusive) develop a reduction in nerve-evoked ACh release at the neuromuscular junction (Takamori, Hamada, Komai, Takahashi & Yoshida, 1994). This proposition contrasts with the Western blot analysis indicating that none of the sera from fourteen LES patients, examined for their ability to recognize the nerve terminal proteins, reacted with pure recombinant synaptotagmin or syntaxin (Hajela & Atchison, 1995). Our observation that ionomycin triggers the equal amount of ACh release from synaptosomes incubated with LES and control serum (Fig. 4) also fails to support this hypothesis. Thus it remains to be resolved whether synaptotagmin is indeed a co-molecular target of LES antibodies and/or plays any role in LES pathogenesis.

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