Lambert-Eaton Antibodies Inhibit Ca$^{2+}$ Currents But Paradoxically Increase Exocytosis during Stimulus Trains in Bovine Adrenal Chromaffin Cells

Kathrin L. Engisch,1 Mark M. Rich,2 Noah Cook,1 and Martha C. Nowycky1

1Department of Neurobiology and Anatomy, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19129, and 2Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Lambert-Eaton myasthenic syndrome (LEMS) is an autoimmune disease that affects neurotransmitter release at peripheral synapses. LEMS antibodies inhibit Ca$^{2+}$ currents in excitable cells, but it is not known whether there are additional effects on stimulus-secretion coupling. The effect of LEMS antibodies on Ca$^{2+}$ currents and exocytosis was studied in bovine adrenal chromaffin cells using whole-cell voltage clamp in perforated-patch recordings. Purified LEMS IgGs from five patients inhibited N- and P/Q-type Ca$^{2+}$ current components to different extents. The reduction in Ca$^{2+}$ current resulted in smaller exocytotic responses to single depolarizing pulses, but the normal relationship between integrated Ca$^{2+}$ entry and exocytosis (Engisch and Nowycky, 1996) was preserved. The hallmark of LEMS is a large potentiation of neuromuscular transmission (Lambert and Elmqvist, 1971; Tim and Sanders, 1994; Maddison et al., 1998). By analogy it has been suggested N- and P/Q-type Ca$^{2+}$ channels with Ca$^{2+}$ channel toxins in chromaffin cells do not

Patients with the Lambert-Eaton myasthenic syndrome (LEMS) have a defect in neuromuscular transmission, thought to be caused by antibody-mediated downregulation of presynaptic calcium channels (Vincent et al., 1989; Engel, 1991; Sher et al., 1993; Lennon et al., 1995). There are two primary changes in neuromuscular function in LEMS (Elmqvist and Lambert, 1968; Cull-Candy et al., 1980): (1) reduction of release evoked by a single stimulus and (2) unusual facilitation during repetitive stimulation. These behaviors are reminiscent of endplate potentials (EPPs) recorded under conditions of low external Ca$^{2+}$ ([Ca$^{2+}$]$_{o}$) and/or high [Mg$^{2+}$]$_{o}$, (for review, see Magelby, 1987). Facilitation is traditionally attributed to Ca$^{2+}$ accumulation during the stimulus train (Katz and Miledi, 1968). By analogy it has been suggested that facilitation in LEMS is also caused by Ca$^{2+}$ accumulation (Lambert and Elmqvist, 1971; Tim and Sanders, 1994; Maddison et al., 1998).

In addition to inhibition of motor nerve terminal Ca$^{2+}$ currents (Smith et al., 1995), LEMS antibodies disrupt the regular arrangement of active zone particles (Fukunaga et al., 1982; Engel, 1991). Loss or disorganization of active zones could affect the Ca$^{2+}$ dependence of neurotransmitter release. Ca$^{2+}$-dependent exocytosis might also be impaired if other synaptic proteins, such as synaptotagmin, are targets of LEMS antibodies [Takahashi et al. (1991); Levelque et al. (1992); Yoshida et al. (1992); Takamori et al. (1994, 1995); Charvin et al. (1997); but see Hajela and Atchison (1995)].

The adrenal chromaffin cell is frequently used for studies of Ca$^{2+}$-secretion coupling (Trifaro et al., 1993; Morgan and Burgoyne, 1997; Burgoyne and Morgan, 1998). Changes in membrane capacitance can be used in these cells to monitor exocytosis of large dense-cored vesicles (Neher and Marty, 1982). We have shown previously that in perforated-patch recordings, exocytosis evoked by single depolarizations is a function of integrated Ca$^{2+}$ entry, raised to the ~1.5 power (Engisch and Nowycky, 1996). During repetitive stimulation, chromaffin cells display activity-dependent behaviors, such as increases in the Ca$^{2+}$-exocytosis relationship (“enhancement”) or decreases in the Ca$^{2+}$-exocytosis relationship (“depression”) (Engisch et al., 1997).

LEMS antibodies inhibit Ca$^{2+}$ currents in bovine chromaffin cells (Kim and Neher, 1988; Viglione et al., 1992; Blandino and Kim, 1993) but have no effect on exocytosis elicited by intracellular perfusion with buffered Ca$^{2+}$ solutions (Kim and Neher, 1988). This suggests that LEMS antibodies do not act directly on the Ca$^{2+}$-dependent fusion machinery. However, the effect of LEMS antibodies on exocytosis evoked by depolarization-induced Ca$^{2+}$ entry is not known. Simple inhibition of Ca$^{2+}$ channels with Ca$^{2+}$ channel toxins in chromaffin cells does not
change the Ca$^{2+}$ dependence of exocytosis evoked by single pulses (Engisch and Nowycky, 1996). On the other hand, small Ca$^{2+}$ current integrals are more likely to induce enhancement during stimulus trains (Engisch et al., 1997). Effects of LEMS antibodies on neurotransmission may be caused entirely by inhibition of Ca$^{2+}$ currents, or there may be additional actions of LEMS antibodies on stimulus-secretion coupling. To examine these possibilities we treated bovine adrenal chromaffin cells with five LEMS IgGs and determined Ca$^{2+}$–exocytosis relationships during single pulses and stimulus trains.

**MATERIALS AND METHODS**

**Chromaffin cell culture.** Adrenal chromaffin cells were prepared from adult bovine adrenal glands by collagenase digestion (0.02%) and purification on a Percoll gradient (Pharmacia, Piscataway, NJ), as described in Vitale et al. (1991). Cells were plated on 12-mm-diameter collagen-coated glass coverslips (8.4 × 10$^5$ cells/coverslip) in a culture medium consisting of DMEM, supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS), antibiotics (penicillin, 0.01%; streptomycin, 0.01%; and gentamycin, 0.001%), and mitotic inhibitors (cytosine arabinoside, 10 μM; 2-deoxy-D-glucose, 10 μM). Cells were used between day 3 and day 7 in vitro and were fed on day 3 and day 6.

**Purification of LEMS antibodies.** IgGs were purified by running human sera over a protein G column (Pharmacia, Piscataway, NJ) and eluting the bound IgG molecules according to the manufacturer’s instructions. IgGs were concentrated in Dulbecco’s PBS to stock concentrations of 50–100 mg/ml by centrifugation in a 10 kDa cutoff Centricon (Amicon, Beverly, MA). IgG concentrations were determined using the Lowry method, with bovine serum albumin as a standard. It was assumed that all protein in the purified sample was IgG. Stocks and sera were kept frozen at –80°C. Care was taken not to subject IgGs to more than two freeze–thaw cycles.

**Treatment of chromaffin cell cultures with purified IgG.** Stock IgG was added to individual cultures at a final concentration of 1–2 mg/ml. Typically 4–8 μl of stock solution was added to a culture well containing 400 μl of culture medium. Cells were assayed after 24 or 48 hr of incubation in IgG. For a 48 hr treatment, fresh stock IgG was added to the culture 24 hr after the initial addition. At most, two IgGs were tested on cells from a single culture (culture = cells from one bovine adrenal gland), and cells in untreated dishes of the same culture served as controls. IgGs from non-disease subjects were tested in the same way, with untreated cells from the same culture as controls. Each IgG was tested on cells from a minimum of three cultures (range, three to seven cultures). No dramatic differences were observed between 24 or 48 hr IgG incubations, or between 1 or 2 mg/ml IgG, so these data have been pooled in the final analysis. The majority of data were obtained using 1 mg/ml for 48 hr.

**Electrophysiological solutions and recording conditions.** Individual glass coverslips were transferred to a chamber perfused with extracellular recording solution at a rate of 1–2 ml/min. Extracellular solution contained (in mM): 135 Cs-glutamate, 10 HEPES (pH 7.5) or 10 morpholino propane sulfonic acid (pH 7.2), 9.5 NaCl, and 0.5 Na$_4$BAPTA (pH adjusted to 7.2 with CsOH, 10–30 min at 37°C) for acute recordings and 30–310 mM CsOM (adjusted with mannitol). Amphotericin B was included in the pipette solution as follows. Amphotericin B was prepared as a stock solution (125 mg/ml) in dimethyl sulfoxide (DMSO) by sonication and was kept in the dark at room temperature for up to 2 hr. Stock amphotericin B solution was added to intracellular solution at a final concentration of 0.5 mg/ml and dispersed by homogenization with a Pro-250 Homogenizer (Pro Scientific, Monroe, CT) for 5–10 sec. Because amphotericin B interferes with seal formation, patch pipettes were pre-dipped (10–15 sec) in amphotericin B-free intracellular solution and backfilled with amphotericin B-containing solution.

CsOH was obtained from ICN Biochemicals (Aurora, OH), amphotericin B and glutamic acid were from Calbiochem (La Jolla, CA), Na$_4$BAPTA was from Molecular Probes (Eugene, OR), and DMSO was from Aldrich (Milwaukee, WI). Culture media and PBS were purchased from Life Technologies (Grand Island, NY). Collagenase was obtained from Worthington (Lakewood, NJ), and FBS was from Biocell (Rancho Dominguez, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Capacitance detection.** Capacitance measurements were performed in perforated-patch whole-cell voltage-clamp recordings using a modified List EPC-7 patch-clamp amplifier and a software-based phase-tracking algorithm (Joshi and Fernandez, 1988; Fidler and Fernandez, 1989). A sine wave stimulus (40 mV peak-to-peak amplitude, 1400 Hz) was added to a holding potential of –90 mV. Orthogonal phase angles for measuring capacitance and conductance were calculated at the beginning of each capacitance trace (trace = 18 sec) by measuring changes in sine wave current produced by transiently connecting a 500 kΩ resistor in series with ground. Ten sine waves were averaged for each capacitance and conductance point. The time resolution was 18 msec/point (486 IBM clone personal computer). Data acquisition was initiated when the access conductance increased to 70 nS. Capacitance changes were calibrated by electronic displacement of 100 pF in the capacitance compensation circuitry of the patch clamp. The amplitude of a stimulus-evoked membrane capacitance (Cm) increase was determined from the difference between a 10 point average (–180 msec) before depolarization and the 10 point average after return to capacitance recording after the depolarizing stimulus. Cells were stimulated every 2 min to allow complete recovery of Ca$^{2+}$ currents from inactivation. Often the Cm response after a large stimulus was followed by a faster depolarization (20 msec), so this time was not included in the analysis of current integrals. The current integrals are more likely to induce enhancement or depression of the Ca$^{2+}$ current than either cumulative exocytosis or current amplitudes. The baseline current was subtracted from the peak current before integration. Current integrals were measured relative to the Cm response to a single depolarization from a holding potential of 50 mV to 0 mV and were averaged over 40 msec (20 pulses). The cumulative exocytotic response was plotted as a function of cumulative Ca$^{2+}$ entry, calculated by integrating each individual current and summing for all the pulses in a train.

Classifications of enhancement or depression of the Ca$^{2+}$–exocytosis relationship were made when the amount of exocytosis during a train was larger or smaller, respectively, than that predicted by the single-pulse standard curve. A response was classified as “enhanced” if the amount of...
Table 1. Unusual responses evoked by stimulus trains in cells treated with LEMS IgGs

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Endocytosis</th>
<th>Docked</th>
<th>Delayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 msec train</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10 msec train</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>40 msec train</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

A response was classified as endocytosis if there was a net negative change in CM during the stimulus train. A response was classified as docked if there were large CM changes evoked by the initial pulses in a train, followed by cessation of exocytosis (Engisch and Nowycky, 1996). A response was classified as delayed if exocytosis was initially below the standard single-pulse Ca\(^{2+}\)-exocytosis relationship (see Materials and Methods; Figs. 3–5, S, dotted curves), but significant exocytosis was evoked by later pulses in the train. The total number of trains for each protocol is given in Figure 6 legend.

Table 2. LEMS patient data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cancer</th>
<th>Age/sex</th>
<th>Initial CMAP (mV)</th>
<th>Post-exercise (mV)</th>
<th>% Increment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>35/F</td>
<td>0.98</td>
<td>7.45</td>
<td>760</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>71/M</td>
<td>0.55</td>
<td>2.70</td>
<td>490</td>
</tr>
<tr>
<td>4</td>
<td>SCLC</td>
<td>60/M</td>
<td>2.90</td>
<td>6.67</td>
<td>230</td>
</tr>
<tr>
<td>5</td>
<td>SCLC</td>
<td>69/M</td>
<td>0.67</td>
<td>2.75</td>
<td>410</td>
</tr>
<tr>
<td>7</td>
<td>SCLC</td>
<td>63/M</td>
<td>0.25</td>
<td>6.50</td>
<td>2600</td>
</tr>
</tbody>
</table>

Samples of serum were obtained from five patients diagnosed with LEMS based on initial CMAP amplitude in abductor pollicis brevis muscles and percentage increment after 15 sec of voluntary maximal contraction (Post-exercise). Three of the patients had small-cell lung cancer (SCLC); at the time of this study no cancer had been detected in two of the patients. Normal CMAP amplitude in abductor pollicis brevis is ~7 mV (Kimura, 1989).
of the total current, which may explain why the effect of LEMS 4 IgG did not reach statistical significance for total Ca\(^{2+}\) entry (Fig. 2A).

The heterogeneous effects of LEMS IgGs on Ca\(^{2+}\) channels are consistent with most previous reports (Kim and Neher, 1988; Blandino and Kim, 1993; Grassi et al., 1994; Blandino et al., 1995; Viglione et al., 1995; Garcia and Beam, 1996; Garcia et al., 1996; Magnelli et al., 1996; Meriney et al., 1996). Here we show that IgG from a single patient (LEMS 1 and LEMS 3) can act on two calcium channel subtypes (Fig. 2B,C). On the other hand, an individual patient IgG can very specifically target a single Ca\(^{2+}\) current component. LEMS 5 strongly inhibited the P/Q-type component (Fig. 2C, right), similar to the application of 1 μM agatoxin IVA (Fig. 1Aii), but had no effect on the N-type current component (Fig. 2B). The more consistent inhibition of the P/Q-type current component is in agreement with binding studies showing that >80% of Lambert-Eaton patients have high anti-P/Q titers, whereas only 40% have high anti-N titers (Lennon et al., 1995; Motomura et al., 1997).

There was a correspondence between Ca\(^{2+}\) current inhibition by LEMS IgGs in chromaffin cells and the severity of the disease, based on measurements of the initial CMAP (Table 2). Total Ca\(^{2+}\) entry in chromaffin cells correlated with initial CMAP amplitude (\(r = 0.89\), assuming a value of 7 mV for control and normal IgG) (Kimura, 1989). This correlation appears to be attributable to effects on the P/Q-type Ca\(^{2+}\) channel, because the \(r\) value for P/Q-type current amplitude versus initial CMAP amplitude was 0.88 but for N-type Ca\(^{2+}\) the current amplitude was 0.30.

LEMS antibodies do not change the basal Ca\(^{2+}\)-exocytosis relationship observed during single step depolarizations

In perforated-patch recordings of adult bovine adrenal chromaffin cells, exocytosis evoked by single step depolarizations has a simple but nonlinear dependence on integrated Ca\(^{2+}\) entry (Engisch and Nowycky, 1996; Engisch et al., 1997) (see Materials and Methods): \(\Delta C_m = g \times (Q_{cap})^n\), where \(\Delta C_m\) is the increase in membrane capacitance, \(Q_{cap}\) is the integral of the Ca\(^{2+}\) current in picocoulombs, \(g\) is a proportionality constant, and \(n\) is the power.

Figure 1. Pharmacological and kinetic dissection of inward current in adult bovine adrenal chromaffin cells. Ai, Perfusion with 1 μM \(\omega\)-conotoxin GV1A selectively inhibits the peak inward current evoked by a 320 msec depolarization from −90 to +20 mV. Aii, Perfusion with 1 μM \(\omega\)-agatoxin IVA almost completely inhibits inward current measured at the end of a 320 msec voltage step (different cell from i). Aiii, Plateau current is measured at the end of a 320 msec voltage step. Difference current is obtained by subtracting this amount from the peak inward current. In this and subsequent figures, the first rapid inward current component is the Na\(^+\) current. B, Amplitude of the difference current for cells before toxin application (control) or after perfusion or preincubation in 1 μM the indicated toxins, alone or together (both). A remaining component of difference current after application of both toxins may be carried by another calcium channel subtype. Numbers above bars indicate number of cells. C, Amplitude of the plateau current for cells before and after exposure to calcium channel toxins. Although \(\omega\)-conotoxin does not significantly inhibit the plateau current when applied alone, there is an additive effect when the toxins are co-applied, suggesting that some overlap of channel subtypes contribute to this component. Data from the same cells as in B.
Engisch et al. (1997) For the average curve, which we will refer to as the standard Ca\(^{2+}\)–exocytosis relationship, \(g = 0.147\) and \(n = 1.49\). This relationship has been plotted as a dashed curve in Figures 3-5 and 8.

To determine whether the single-pulse Ca\(^{2+}\)–exocytosis relationship was affected by LEMS antibodies, exocytosis was evoked by single depolarizations. Pulse duration or test potential was varied to sample a range of Ca\(^{2+}\) entry values. In an untreated chromaffin cell, single depolarizations evoked larger capacitance increases than the same voltage steps in a cell treated with LEMS 3 IgG (Fig. 3A: Control: 1, 2; LEMS 3: 3, 4). The relationship between integrated Ca\(^{2+}\) entry (\(Q_{Ca}\)) and amount of exocytosis (\(D_{Cm}\)) is shown in Figure 3B for the two cells. Both sets of data lie close to the standard input–output relationship (dashed curve), indicating that the Ca\(^{2+}\) dependence of exocytosis was not altered by treatment with LEMS 3 IgG. All values in the LEMS 3-treated cell were simply shifted down the input–output relationship to a region of small responses.

Similar experiments were performed in cells treated with five different LEMS IgGs and IgGs from non-disease controls. Re-
responses were binned by Ca\(^{2+}\) current integrals and averaged for each IgG (Fig. 4). A plot of the standard Ca\(^{2+}\)–exocytosis relationship (dashed curve) is overlaid on the data. The average responses in IgG-treated cells lie close to the standard curve, regardless of which channel type(s) was affected. In summary, five LEMS IgGs that differentially affect N- and P/Q-Ca\(^{2+}\) channel subtypes reduce exocytosis but do not change the single-pulse Ca\(^{2+}\)–exocytosis relationship.

**LEMS antibodies promote activity-dependent enhancement during stimulus trains**

The key diagnostic feature of the Lambert-Eaton myasthenic syndrome is a large potentiation of neuromuscular transmission after high-frequency repetitive stimulation (Table 2). We have previously described two types of modulation of the Ca\(^{2+}\)–exocytosis relationship that can occur in bovine chromaffin cells during repetitive stimulation (Engisch et al., 1997). Some trains evoke exocytosis that has the same relationship with integrated Ca\(^{2+}\) entry as exocytosis stimulated by single pulses (Fig. 5Ai,Bi). Other trains evoke exocytosis that shows potentiation of the Ca\(^{2+}\)–exocytosis relationship (Fig. 5Ai,Bi). Trains in a third group evoke much less exocytosis than expected from the single pulse Ca\(^{2+}\)–exocytosis relationship and are classified as depressed (Fig. 5Aiii,Biii).

In untreated cells the likelihood of obtaining enhancement, depression, or a standard input–output relationship during a stimulus train is correlated with the amount of Ca\(^{2+}\) entry during the first pulse of the train (Engisch et al., 1997). Enhancement was observed in >30% of trains made up of 5 msec pulses (Fig. 6, CONTROL, 5 ms, white section). In contrast, a train of 40 msec pulses usually produced depression (~90% of trains) (Fig. 6, CONTROL, 40 ms, black section), and enhancement was only rarely observed. The distribution of response behaviors for trains of 10 msec pulses was intermediate between that for 5 and 40 msec pulses. In addition, when compared in the same cell, a train of 5 msec pulses was almost always more efficacious than a train of 40 msec pulses, unless the two protocols evoked responses with the same Ca\(^{2+}\)–exocytosis relationship (Engisch et al., 1997).

We examined whether the reduction in Ca\(^{2+}\) entry caused by treatment with LEMS antibodies would lead to a greater percent of trains with enhancement. We found that the percentage of trains inducing enhancement was increased for all pulse protocols (Fig. 6; compare white sections, CONTROL vs LEMS). A greater proportion of trains with enhancement resulted not only from decreases in the number of depressed responses (black sections) but also from decreases in standard responses (cross-hatched sections). There were some unusual response behaviors after exposure to LEMS antibodies that could not be classified into the categories used for controls, but these were relatively rare (Other, 7–14%; striped sections; for details, see Figure 6 legend and Table 1). In summary, it appears that decreasing Ca\(^{2+}\) entry at any pulse duration led to an increase in the probability of enhance-
A subset of LEMS IgGs promotes activity-dependent enhancement even after effects of Ca\(^{2+}\) current inhibition have been taken into account

We grouped trains by amount of Ca\(^{2+}\) entry, rather than by pulse duration, to compare responses from controls and LEMS-treated cells after normalizing for the effects of LEMS antibodies on Ca\(^{2+}\) currents. This procedure will reveal whether there are any additional changes in activity-dependent behaviors after exposure to LEMS IgGs. We grouped trains into three ranges based on the amount of Ca\(^{2+}\) entry during the first pulse of the train: low, middle, and high.

In the middle and high ranges, data from the five LEMS IgGs were pooled, because the number of trains was insufficient for adequate comparison of results for individual IgGs. In control cells, essentially all trains in the high range (Ca\(^{2+}\) entry >6 \(\times\) 10\(^7\) ions or 19 pC) evoked a depressed response [67/68; compare Engisch et al. (1997), their Fig. 4]. Similarly, depression occurred in the vast majority of trains from LEMS-treated cells that fell in the high range (24/25 trains). In the middle range (Ca\(^{2+}\) entry between 2 and 6 \(\times\) 10\(^7\) ions, or 6.4 and 19 pC), the percentage of trains with depression was slightly lower in LEMS-treated cells compared with controls (54 vs 69%). These results indicate that the ability of large Ca\(^{2+}\) loads to induce depression is not substantially altered by treatment with LEMS IgGs.

The probability of obtaining enhanced or standard responses was increased as pulse duration was shortened in control cells. The distribution of response behaviors evoked in control cells by stimulus trains within the low range of Ca\(^{2+}\) entry (Q\(_{Ca}\) <2 \(\times\) 10\(^7\) ions or 6.4 pC) is illustrated in Figure 7A [Engisch et al. (1997), data reproduced from first two bins of their Fig. 4]. Enhancement, a standard Ca\(^{2+}\)–exocytosis relationship, and depression are approximately equally likely, with a slight trend toward enhancement. In this Ca\(^{2+}\) entry range there were sufficient numbers of trains in LEMS-treated cells so that each IgG could be separately examined. The proportion of trains with enhancement was clearly not increased in cells treated with non-LEMS control IgG (Fig. 7B), LEMS 1 (Fig. 7C), or LEMS 5 (Fig. 7F). In cells treated with LEMS 7 there were no depressed responses evoked by trains (Fig. 7G), but because so few depressed responses are expected, this change was not statistically significant. For two of the LEMS IgGs, the distribution of secretory behaviors was different from the expected values. Enhancement was observed in ~70% of the trains in cells treated with LEMS 3 (Fig. 7D) or LEMS 4 (Fig. 7E), almost twice the normal.
The shift to greater numbers of trains with enhancement was statistically significant at the 0.05 (LEMS 3) and 0.01 (LEMS 4) levels (Pearson’s χ² test). Finally, the shifts occurred although average Ca²⁺ current integrals were not statistically different from the average integral for non-disease control IgG (LEMS 3 IgG, 2.7 ± 0.3 pC; LEMS 4 IgG, 3.2 ± 0.3 pC; normal IgG, 3.5 ± 0.3 pC). Thus, IgGs from a subset of patients appear to make conditions unusually favorable for activity-dependent enhancement, through a mechanism other than inhibition of Ca⁡²⁺ currents.

In summary, our data indicate that a chromaffin cell treated with LEMS IgG will have reduced exocytosis in response to a single stimulus, but will be more likely to show activity-dependent enhancement of exocytosis during a train. This situation closely resembles the neuromuscular defect in the Lambert-Eaton myasthenic syndrome. In Figure 8, exocytosis evoked by single depolarizations is compared with exocytosis evoked by a train in an individual chromaffin cell exposed to LEMS 3 IgG. Single 160 msec depolarizations evoked less exocytosis than the stimulus train, when similar amounts of Ca²⁺ entry were compared. Thus, during repetitive stimulation a reduction in Ca²⁺ entry by LEMS antibodies does not necessarily lead to a decrease in exocytosis.

Instead there may be a paradoxical increase in the amount of release attributable to the occurrence of activity-dependent enhancement.

**DISCUSSION**

We studied the effect of LEMS IgGs on Ca²⁺ currents and depolarization-evoked exocytosis in bovine adrenal chromaffin cells. Three IgGs inhibited only P/Q-type Ca²⁺ current, and two additionally affected N-type Ca²⁺ current, in agreement with studies suggesting that LEMS antibodies can target multiple sites (Johnston et al., 1994; Takamori et al., 1997; Katz et al., 1998; Verschuuren et al., 1998) (also see Results). Our findings disagree with the suggestion that N-type calcium channels are not functionally affected by LEMS antibodies (Pinto et al., 1998). There are several possible reasons for the difference in results. First, the effect we observe was confined to two of five IgGs tested. Second, the small effect on N-type Ca⁡²⁺ current (maximum 24%) might have been missed in the K⁡⁺-stimulated [Ca⁡²⁺], measurements used by Pinto et al. (1998). In any case, the effect on P/Q-type Ca⁡²⁺ current appears to be responsible for the clinical deficits. Total Ca²⁺ entry and P/Q-type current amplitude roughly correlated with the size of CMAPs in the five LEMS patients,
[Mg$^{2+}$], (Dodge and Rahaminoff, 1967; Hubbard et al., 1968; Cooke et al., 1973; Cull-Candy et al., 1976). A reduction in the number of Ca$^{2+}$ channels at the terminal should leave the power unaltered but shift the relationship to the right, because higher levels of [Ca$^{2+}$], are required to evoke the same amount of release. The power of the relationship between [Ca$^{2+}$], and release at LEMS patient NMJs appeared to be decreased to ~1.5 (Cull-Candy et al., 1980). Although the authors concluded that LEMS is associated with a lower Ca$^{2+}$ sensitivity of the release process, the 1.5-power relationship was probably the result of focusing on the physiological range of Ca$^{2+}$ and Mg$^{2+}$ concentrations in that study. In experiments in high [Mg$^{2+}$], at the mouse NMJ after passive transfer of LEMS, a power dependence of 3.9 was observed, and the relationship between endplate potentials and [Ca$^{2+}$], was indeed shifted to the right (Lang et al., 1987).

The dependence of transmitter release on Ca$^{2+}$ influx, rather than [Ca$^{2+}$],, cannot be directly examined at the NMJ because it is difficult to measure Ca$^{2+}$ currents in the motorneuron terminal. This question can be addressed in control and LEMS-treated bovine adrenal chromaffin cells. Exocytotic responses evoked by single depolarizations in LEMS-treated cells closely followed the relationship between Ca$^{2+}$ influx and exocytosis that was derived in control cells (Engisch and Nowycky, 1996). Similarly, in whole-cell capacitance recordings of H146 cells (a small-cell lung cancer cell line), exocytosis evoked by single long depolarizations was reduced in proportion to reductions in plateau current by either LEMS antibody treatment or exposure to α-agatoxin IVA (Viglione et al., 1995). These results support the suggestion that the Ca$^{2+}$ dependence of release is preserved in LEMS.

The hallmark of LEMS is a small CMAP that facilitates after repetitive stimulation. CMAP measures the sum of action potentials (APs) generated in the muscle by acetylcholine released during nerve stimulation. In controls the CMAP amplitude does not facilitate during repetitive stimulation, but this could be because 100% of muscle fibers are already firing APs. A more sensitive measure of presynaptic activity is the EPP. During high-frequency stimulation, EPPs decrease at normal NMJs (Elmqvist and Quastel, 1965) but increase at NMJs of LEMS patients (Elmqvist and Lambert, 1968). Facilitation is also observed at normal mammalian NMJs under conditions in which the initial response is reduced, usually by lowering [Ca$^{2+}$], and/or raising [Mg$^{2+}$], (Del Castillo and Katz, 1954). Katz and Miledi (1968) proposed the residual Ca$^{2+}$ hypothesis to explain activity-dependent facilitation of transmitter release: when [Ca$^{2+}$], is low, insufficient Ca$^{2+}$ ions enter during a single AP to trigger maximal release but accumulate during a train, and each successive AP triggers more release. More recent modifications of this hypothesis postulate the existence of a facilitation site that senses accumulated Ca$^{2+}$, distinct from the exocytosis trigger (Kamiya and Zucker, 1994; Zucker, 1996). Because the LEMS antibodies inhibit Ca$^{2+}$ currents and reduce evoked release, the abnormal facilitation in LEMS has been attributed to Ca$^{2+}$ accumulation (Lambert and Elmqvist, 1971; Tim and Sanders, 1994).

Our previous data in chromaffin cells suggest that activity-dependent facilitation may be caused by more than the simple accumulation of Ca$^{2+}$ ions beneath the plasma membrane. Ca$^{2+}$ accumulation should be increased when pulse interval is shortened, but this manipulation prevented the development of enhancement in chromaffin cells (Engisch et al., 1997). In addition, although greater Ca$^{2+}$ accumulation would be expected for trains of longer duration pulses at the same frequency, these protocols...
induced depression. Depression is usually attributed to vesicle depletion (Elmqvist and Quastel, 1965; Thies, 1965; Zucker, 1989). In chromaffin cells depression is not caused by depletion because it occurs after a smaller amount of exocytosis than is evoked by a single long depolarization in the same cell (Engisch et al., 1997). We concluded that in chromaffin cells, specific patterns of Ca$^{2+}$ entry induce a change in the Ca$^{2+}$ sensitivity of the secretory process.

Exposure of chromaffin cells to LEMS antibodies could have produced any one of the following effects on exocytosis evoked by stimulus trains. (1) If the only action of LEMS antibodies is to decrease Ca$^{2+}$ entry, the likelihood of depression should decrease and that of enhancement increase, for the same stimulus parameters (duration, pulse interval); (2) decreased Ca$^{2+}$ entry could result in less exocytosis during a train, as it does during single pulses; and (3) if LEMS antibodies target proteins other than Ca$^{2+}$ channels, novel behaviors could occur, or depression or enhancement may be either increased or reduced beyond the effects expected for changes in Ca$^{2+}$ entry.

For three protocols in cells exposed to LEMS IgGs (trains of 5, 10, or 40 msec pulses, 200 msec intervals), secretory behaviors shifted from fewer depressed responses to more enhanced responses, an effect that is expected for a simple decrease in Ca$^{2+}$ entry. Enhancement in LEMS-treated cells was not caused by Ca$^{2+}$ accumulation because it resembled enhancement in untreated cells, being abolished rather than increased when pulses...

---

**Figure 7.** Distribution of secretory behaviors evoked by trains with small amounts of Ca$^{2+}$ entry for the first pulse of the train. A, Control distribution, taken from Engisch et al. (1997). Low range of Ca$^{2+}$ entry: $Q_{ca} < 6.4$ pC. Percentage of trains in a large sample ($n = 106$) with the indicated secretory behaviors. This distribution was used to give expected values for the sample sizes in treated groups. B–F. Number of trains in each category for cells treated with IgGs. There is an unusually large number of enhanced responses in cells treated with LEMS 3 IgG (D) and LEMS 4 IgG (E). LEMS 1, 5, and 7 had normal distributions of secretory behaviors. Other responses were not included in the statistical comparison; the total number of trains was taken after subtracting any Other trains. * $p < 0.05$, ** $p < 0.01$; Pearson’s $\chi^2$ test.

**Figure 8.** Comparison of single pulse responses with a train-evoked response in a LEMS 3-treated chromaffin cell. A, Membrane capacitance trace ($Cm$) recorded during repetitive stimulation with 5 msec depolarizing voltage steps (−90 to +20 mV) in a cell that had been treated for 24 hr with LEMS 3 IgG (1 mg/ml; same cell as in Fig. 3). The inward currents evoked by the first and last depolarization of the train are illustrated below ($I_{Ca}$). B, Cm increases evoked by the stimulus train in A (□) and single 160 msec depolarizations to different test potentials (■; −5, 0, and +20 mV) test pulses from Fig. 3 plotted versus integrated Ca$^{2+}$ entry. The dashed curve is the standard single pulse Ca$^{2+}$-exocytosis relationship (see Materials and Methods).
were applied at higher frequency (K. Engisch and M. Nowycky, unpublished observations). Unusual secretory behaviors did occur in treated cells, but these were rare (~10% of all trains). The process of depression per se was not altered by the antibodies. Trains with large Ca2+ current integrals in LEMS-treated cells caused depressed responses at the expected (>90%) frequency.

Although much of the increase in the probability of activity-dependent enhancement can be explained by the ability of LEMS IgGs to inhibit Ca2+ entry, there appeared to be an additional action on enhancement for two of the five LEMS IgGs. First, the percentage of trains with enhancement was approximately double for cells treated with LEMS 3 or LEMS 4 IgGs, compared with controls within the same narrow range of low Ca2+ entry values. Second, LEMS 4 IgG increased enhancement without substantially inhibiting Ca2+ entry. In conclusion, all five LEMS antibodies increased the probability of activity-dependent enhancement in chromaffin cells. Effects of three of the antibodies could be attributed solely to a reduction in Ca2+ entry. Two of the antibodies appeared to have an additional influence on the enhancement process.

Our results suggest that a possible target of LEMS IgGs, in addition to presynaptic Ca2+ channels, is a protein or complex of proteins important for controlling activity-dependent facilitation. A key finding is that the probability of facilitation was altered by LEMS IgGs without any change in the Ca2+-exocytosis coupling during a single stimulus. This result suggests that components of the secretory machinery modify the release process but are not mandatory participants in the trigger or fusion mechanisms active during a single stimulus. Chromaffin cells are a useful model system to determine the roles of particular proteins in triggering vesicle fusion, controlling the fusion step, and modulating secretory efficacy. It remains to be determined whether the properties of activity-dependent facilitation in chromaffin cells are applicable to the NMJ or other fast synapses.

REFERENCES


Artalejo CR, Dahmer MK, Perlman RL, Fox AP (1991) Two types of Ca2+ currents are found in bovine chromaffin cells: facilitation is due to the recruitment of one type. J Physiol (Lond) 432:681–707.


