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

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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 after high-frequency stimulation. In chromaffin cells, stimulus trains can induce activity-dependent enhancement of the Ca^{2+}–exocytosis relationship. Enhancement during trains occurs most frequently when pulses are brief and evoke very small amounts of Ca^{2+} entry (Engisch et al., 1997). LEMS antibody treatment increased the percentage of trains eliciting enhancement through two mechanisms: (1) by reducing Ca^{2+} entry and (2) through a Ca^{2+}-independent effect on the process of enhancement. This leads to a paradoxical increase in the amount of exocytosis during stimulus trains, despite inhibition of Ca^{2+} currents.

Key words: exocytosis; chromaffin cell; capacitance detection; Ca^{2+}-secretion coupling; Lambert-Eaton myasthenic syndrome; facilitation

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); Leveque 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

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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 (8.4×10^4 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; fluoro-deoxyuridine, 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): 130 NaCl, 2 KCl, 10 glucose, 10 Na-HEPES, 1 $MgCl_2$, 5 *N*-methyl-D-glucamine, and 5 $CaCl_2$, pH adjusted to 7.2 with HCl; 295 mOsm. Experiments were performed at room temperature (21–26°C).

Perforated-patch intracellular solution contained (in mM): 135 Cs-glutamate, 10 HEPES (p_{Ka} 7.5) or 10 morpholino propane sulfonic acid (p_{Ka} 7.2), 9.5 NaCl, and 0.5 Na_4BAPTA (pH adjusted to 7.2 with CsOH, 305–310 mOsm, 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_4BAPTA 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 Dominquez, 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 fF 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 Ca^{2+} load (stimulus train or long duration pulse) was larger than expected from the single pulse Ca^{2+}–exocytosis relationship, even with a 2 min interval between protocols. Therefore, as described previously, a single 40 msec depolarization was always applied after such a stimulus and the response was discarded from analyses (Engisch and Nowycky, 1996).

Analysis of Ca^{2+} currents. Chromaffin cells were stimulated with depolarizations from a holding potential of −90 mV to a test potential of +20 mV, unless noted otherwise. Pulse duration was varied as indicated in the text and figure legends. Ca^{2+} entry, in picocoulombs, was calculated from integration of inward current, using limits that excluded the major portion of Na^+ current. Sampling rate was 50 kHz for 5 msec pulses, 20 kHz for 10 and 40 msec pulses, 5 kHz for 160 msec pulses, and 2.5 kHz for 320 msec pulses; all currents were filtered at 3 kHz. Tetrodotoxin was not included in the extracellular recording solution because of its slowing effect on the Na^+ channel-gating current that can contribute to depolarization-induced capacitance increases unrelated to exocytosis (Horrigan and Bookman, 1993; Chow et al., 1996). Current traces were leak-subtracted before integration and amplitude measurements. End current amplitude (an estimate of "P/Q-type" current) was determined from an average of 20 points immediately preceding termination of the voltage pulse. Difference current amplitude (an estimate of "N-type" current) was calculated from the difference between the peak current (cursor value at peak located by experimenter) and the end current. Differences in Ca^{2+} current amplitudes and integrals were assessed using Student's *t* test (independent, unless noted otherwise).

Derivation of standard curve. The standard curve depicted in Figures 3–5 and 8 represents the average relationship between Ca^{2+} entry and amount of exocytosis, or capacitance increase, for single depolarizations. It was derived by averaging input–output relationships obtained in 27 cells using single step depolarizations to evoke exocytosis, and different methods to vary Ca^{2+} entry (changing duration or test potential, perfusion with a range of extracellular Ca^{2+} concentrations, or application of Ca^{2+} channel toxins.) Plots of exocytosis as a function of the integral of the Ca^{2+} current were fit with the function: $\Delta Cm = g \times (Q_{Ca})^n$, where ΔCm is the amount of exocytosis in femtofarads, Q_{Ca} is the integral of the Ca^{2+} current in picocoulombs, raised to the *n*th power, and *g* is a proportionality constant; *g* and *n* were varied until χ^2 reached a minimum value (Marquardt-Levenberg algorithm in Origin; Microcal, Northampton, MA). The curves from 27 cells were averaged to generate the standard curve, which was fit with $g = 0.147$ and $n = 1.49$.

Classification of Ca^{2+}–exocytosis relationships during stimulus trains. Exocytosis evoked by a train of depolarizing pulses (−90 mV to + 20 mV), 200 msec between pulses, was analyzed by summing exocytosis evoked by each successive pulse to generate a cumulative response. Pulse duration was 5 msec (typically 35 pulses in a train), 10 msec (30 pulses), or 40 msec (20 pulses). The cumulative exocytotic response was plotted as a function of cumulative Ca^{2+} entry, calculated by integrating each inward current and summing for all the pulses in the 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

Protocol	Endocytosis	Docked	Delayed
5 msec train	3	1	0
10 msec train	0	2	1
40 msec train	0	2	3

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 (Seward 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, 8, *dashed 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.

exocytosis was >1.6× the expected value (from standard curve) and as "depressed" if the amount of exocytosis evoked by the train was <0.8× the expected value [see also Engisch et al. (1997)]. A relationship was classified as "standard" if it fell between these boundaries. Differences between distributions of secretory behaviors were assessed using Pearson's χ^2 test.

Definitions of "other" secretory behaviors. After antibody treatment, trains in several cells evoked unusual secretory behaviors (Table 1) that could not be classified into enhanced, standard, or depressed categories as defined in a previous study (Engisch et al., 1997). "Endocytosis" during a train was characterized by a negative slope of capacitance. A "docked" response had very large Cm increases early in the train (far in excess of the amount expected based on the standard Ca^{2+}–exocytosis relationship); after several pulses there was a rapid decline or cessation of exocytosis. This type of behavior has been observed at the beginning of conventional whole-cell recordings before wash out (Seward and Nowycky, 1996). A "delayed" response fell below the standard relationship initially, with significant exocytosis occurring late in the train. This behavior resembles the "threshold" secretory response that occurs late in the recording period in conventional whole-cell experiments (Seward and Nowycky, 1996).

RESULTS

Clinical findings in five LEMS patients

LEMS was diagnosed based on amplitude of the compound muscle action potential (CMAP) in abductor pollicus brevis muscles. CMAPs measured at rest (initial CMAP) and immediately after 15 sec of voluntary contraction (post-exercise) are given in Table 2 for the five LEMS patients in this study.

Both N- and P/Q-type Ca^{2+} currents are inhibited by LEMS antibodies

Ca^{2+} currents recorded in perforated-patch mode in adult bovine adrenal chromaffin cells are carried primarily by two subtypes of Ca^{2+} channels, which can be kinetically distinguished during prolonged (320 msec) depolarizations (Engisch and Nowycky, 1996). A rapidly inactivating current component is inhibited by 1 μM ω-conotoxin GVIA (Fig. 1*Ai,B*). The plateau current (the current remaining at the end of the pulse) is more sensitive to 1 μM ω-agatoxin IVA (Fig. 1*Aii,C*). Greater inhibition of each current component is observed when both toxins are applied together than when a single toxin is applied (Fig. 1*B,C*). This probably reflects the imperfect separation of the inactivating and noninactivating components at a duration of 320 msec. However, ω-agatoxin IVA does not significantly affect the inactivating component, and ω-conotoxin GVIA does not inhibit the plateau component (Fig. 1*B,C*). For convenience we will refer to the conotoxin-sensitive component as N-type and the agatoxin-sensitive component as P/Q-type, although it is becoming clear that toxin sensitivity is not a sufficient criterion for classifying this complex family (for review, see Randall, 1998). L-type channels, including the facilitation Ca^{2+} channel (Artalejo et al., 1990, 1991a,b), do not contribute significantly to whole-cell Ca^{2+} currents in adult bovine adrenal chromaffin cells (Chow et al., 1996; Engisch and Nowycky, 1996; Elhamdani et al., 1998).

Total Ca^{2+} entry integrated over a 320 msec depolarization was significantly inhibited by treatment with four of five Lambert-Eaton IgGs (Fig. 2*A*). Inhibition of Ca^{2+} entry by LEMS IgGs varied in magnitude, with a maximum block of 39% (LEMS 3). One IgG (LEMS 4) did not inhibit total Ca^{2+} entry nor did treatment with IgGs from subjects without LEMS (*IgG*; data for two control IgGs pooled).

To determine the subtypes of Ca^{2+} channels affected by Lambert-Eaton IgGs, we used the kinetic and pharmacological dissection described in Figure 1. Two of the five patient IgGs, LEMS 1 and LEMS 3, significantly reduced the N-type current component compared with values obtained in untreated cells from sister dishes in the same cultures (Fig. 2*B*, *None*). The N-type component is also slightly smaller in cells treated with control IgGs, but this difference was not significantly different from the average in untreated cells. In addition, a paired Student's *t* test between responses of treated and untreated cells on matching experimental days was not significant ($p > 0.4$; $n = 7$ pairs). In contrast, a paired Student's *t* test between values for LEMS1-treated cells and untreated cells from matching experimental days was highly significant ($p < 0.01$; $n = 7$ pairs). The responses of control IgG-treated cells and LEMS IgG-treated cells were not compared directly because the data were obtained in separate experiments. The maximum percentage reduction in N-type current was only ~24% (458 ± 41 pA, LEMS 1, vs 602 ± 30 pA, untreated cells).

Every patient IgG tested, including LEMS 4, significantly inhibited the P/Q-type current (Fig. 2*C*). Maximum inhibition was 52% (74 ± 9 pA, LEMS 5, vs 156 ± 7 pA, untreated cells). Inhibition of P/Q-type current by LEMS 4 was only 17%. In chromaffin cells the N-type component is a much greater fraction

Table 2. LEMS patient data

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

Samples of serum were obtained from five patients diagnosed with LEMS based on initial CMAP amplitude in abductor pollicus 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 pollicus brevis is ~7 mV (Kimura, 1989).

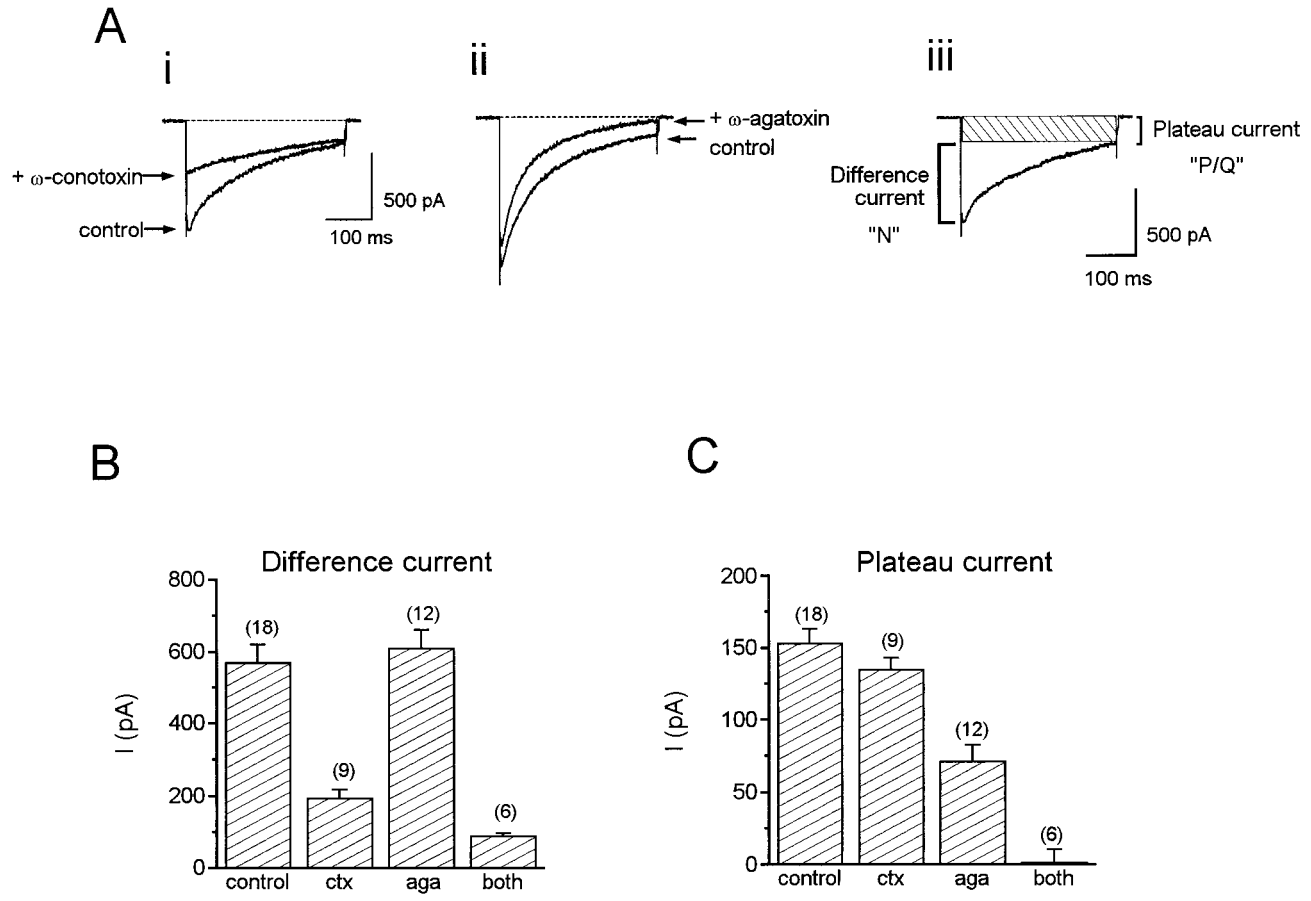

Figure 1. Pharmacological and kinetic dissection of inward current in adult bovine adrenal chromaffin cells. *Ai*, Perfusion with 1 μM ω-conotoxin GVIA selectively inhibits the peak inward current evoked by a 320 msec depolarization from −90 to +20 mV. *Aii*, Perfusion with 1 μM ω-agatoxin IVA almost 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 ω-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*.

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. 2*A*).

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. 2*B,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. 2*C, right*), similar to the application of 1 μM agatoxin IVA (Fig. 1*Aii*), but had no effect on the N-type current component (Fig. 2*B*). 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): $\Delta Cm = g \times (Q_{Ca})^n$, where ΔCm is the increase in membrane capacitance, Q_{Ca} is the integral of the Ca^{2+} current in picocoulombs, g is a proportionality constant, and n is the power

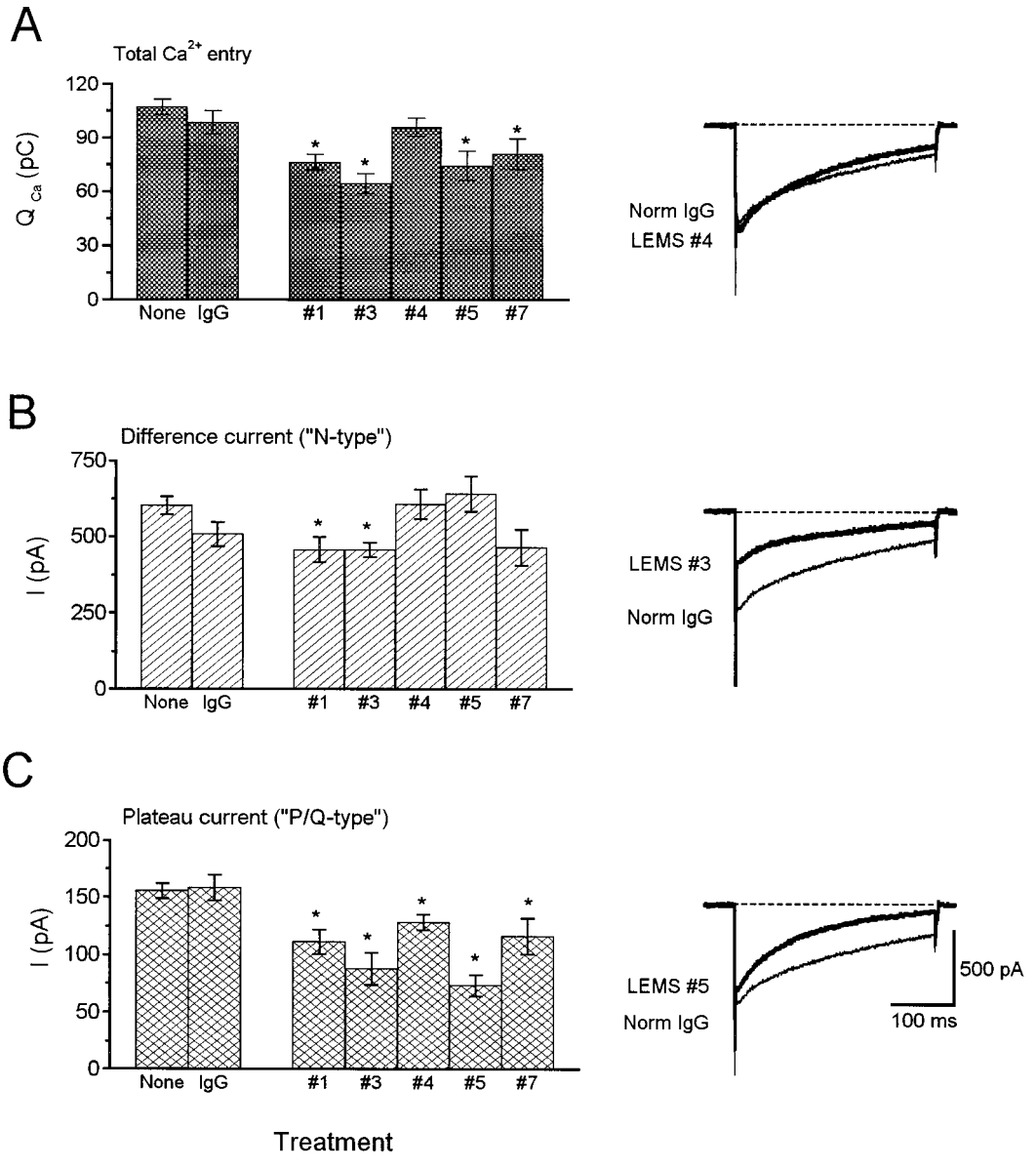

Figure 2. Inhibition of total integrated Ca^{2+} entry, and N- and P/Q-type Ca^{2+} current components, by five LEMS IgGs. *A*, Total Ca^{2+} entry, integrated over a 320 msec depolarizing voltage step from −90 mV to +20 mV, for untreated cells in sister cultures (*None*); cells treated with IgG from non-disease controls (*IgG*; data pooled for cells treated with either of two control IgGs); and cells from five patients with LEMS. Integration excludes the first rapid inward current component, which is carried by Na^+ ions. The number of cells is at least eight for each bar. *Right*, Superimposed inward current traces evoked by a 320 msec voltage step for a cell treated with non-disease IgG (*Norm IgG*, *thin line*) and for another treated with LEMS 4 IgG (*thick line*). Cells were treated with 1 or 2 mg/ml IgG for either 24 or 48 hr (see Materials and Methods). *B*, Amplitude of the difference current (see Fig. 1*Aiii* for description of kinetic components) for IgG-treated cells and cells in untreated sister cultures. *Right*, Inward current trace evoked by a 320 msec depolarization in a cell treated with LEMS 3 IgG (*thick line*), illustrating inhibition of both peak and plateau current, compared with same *Norm IgG* trace (*thin line*) as shown in *A*. *C*, Plateau current amplitude for same cells as in *B*. *Right*, Inward current evoked by 320 msec depolarization in a cell treated with LEMS 5 IgG (*thick line*), illustrating strong inhibition of plateau current with little effect on peak current. *Norm IgG*, Current trace same as in *A* and *B* (*thin line*). $^*p < 0.05$ (Student's *t* test), compared with untreated controls.

(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. 3*A; Control: 1, 2; LEMS 3: 3, 4*). The relationship between integrated Ca^{2+} entry (Q_{Ca}) and amount of exocytosis (ΔCm) is shown in Figure 3*B* 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-

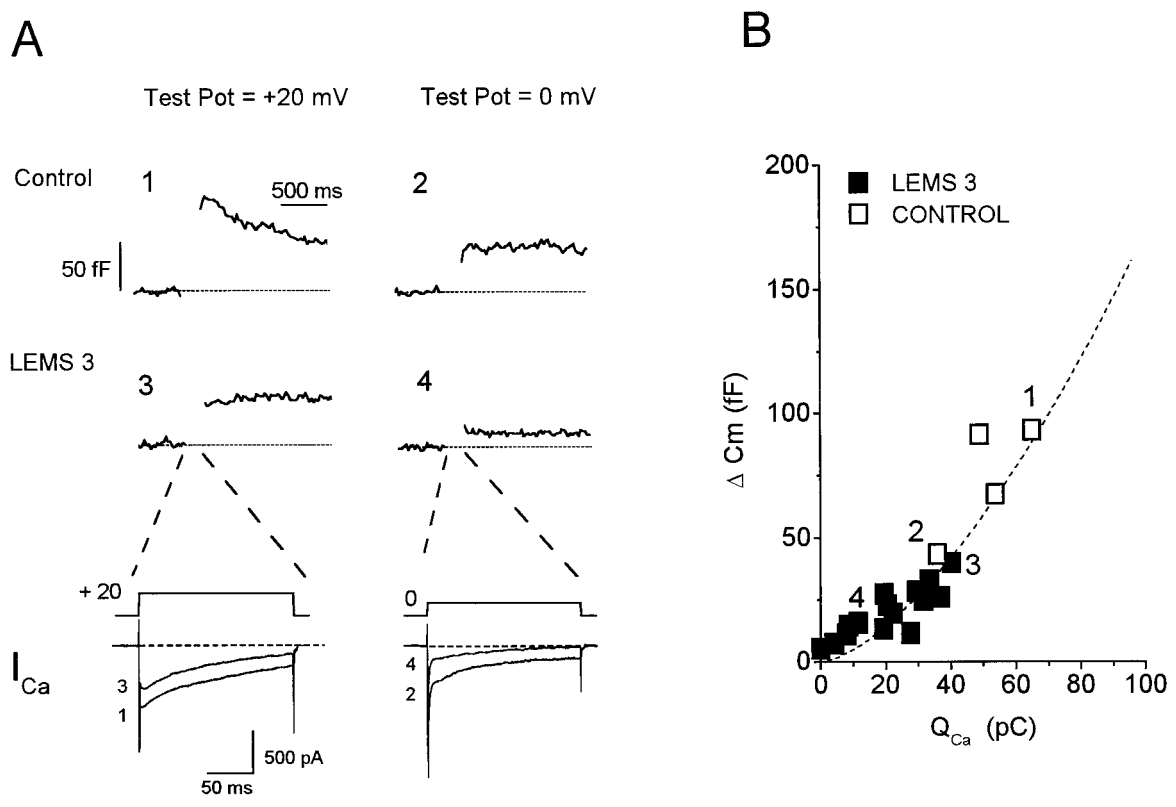

Figure 3. Exocytosis evoked by single depolarizing voltage pulses in a control and a LEMS 3-treated cell. *A*, Cm changes in response to single step depolarizations from −90 mV to the indicated test potentials (*Test Pot*), for a control (untreated) cell and a cell incubated in 1 mg/ml LEMS 3 IgG for 24 hr. Gaps indicate the timing of the depolarization, when capacitance recording is suspended. *Below*, Inward currents evoked by the depolarizations; *numbers* indicate relevant Cm trace. *B*, Cm responses evoked by 160 msec depolarizations to different test potentials, plotted as a function of integrated Ca^{2+} entry, for the cells illustrated in *A*. LEMS 3 IgG indicated by ■; untreated control cell from the same culture indicated by □. The data cluster near the standard curve (*dashes*), a representation of the average relationship during single pulses for adrenal chromaffin cells (see Materials and Methods). The *numbers* adjacent to data points correspond to the numbered traces in *A*.

sponses 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. 5*Aii,Bii*). Other trains evoke exocytosis that shows potentiation of the Ca^{2+}–exocytosis relationship (Fig. 5*Ai,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. 5*Aiii,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-

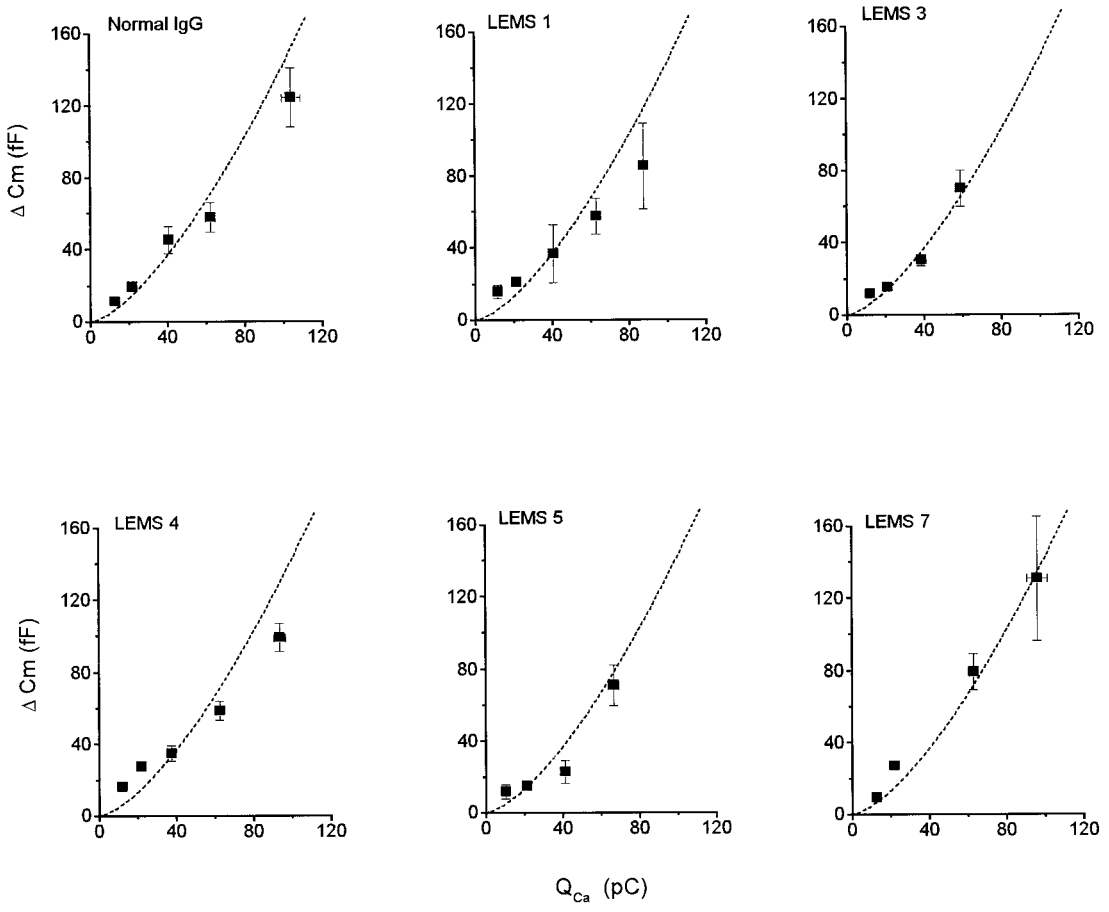

Figure 4. The relationship between exocytosis and integrated Ca^{2+} entry for single depolarizing pulses is unchanged after treatment with control or LEMS IgGs. Cm increases evoked by single depolarizing pulses (40–320 msec in duration, −90 to +20 mV) were binned by amount of Ca^{2+} entry for cells treated with IgG from non-disease controls (*Normal IgG*) and five LEMS patients (bin ranges: below 16 pC, 16–32 pC, 33–48 pC, 49–80 pC, above 80 pC). Two of the IgGs inhibit Ca^{2+} entry to such an extent that only four ranges of Ca^{2+} entry are represented (*LEMS 3*, *LEMS 5*). The points are overlaid on the standard curve (*dashes*) for comparison purposes. Each point is the average of at least eight measurements, except for the values at the largest Ca^{2+} entry bin for LEMS 1 and LEMS 7, which are the average of only five measurements.

ment, at the expense of standard and depressed Ca^{2+}–exocytosis relationships.

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 6×10^7 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 7*A* [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. 7*B*), LEMS 1 (Fig. 7*C*), or LEMS 5 (Fig. 7*F*). In cells treated with LEMS 7 there were no depressed responses evoked by trains (Fig. 7*G*), 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. 7*D*) or LEMS 4 (Fig. 7*E*), almost twice the normal

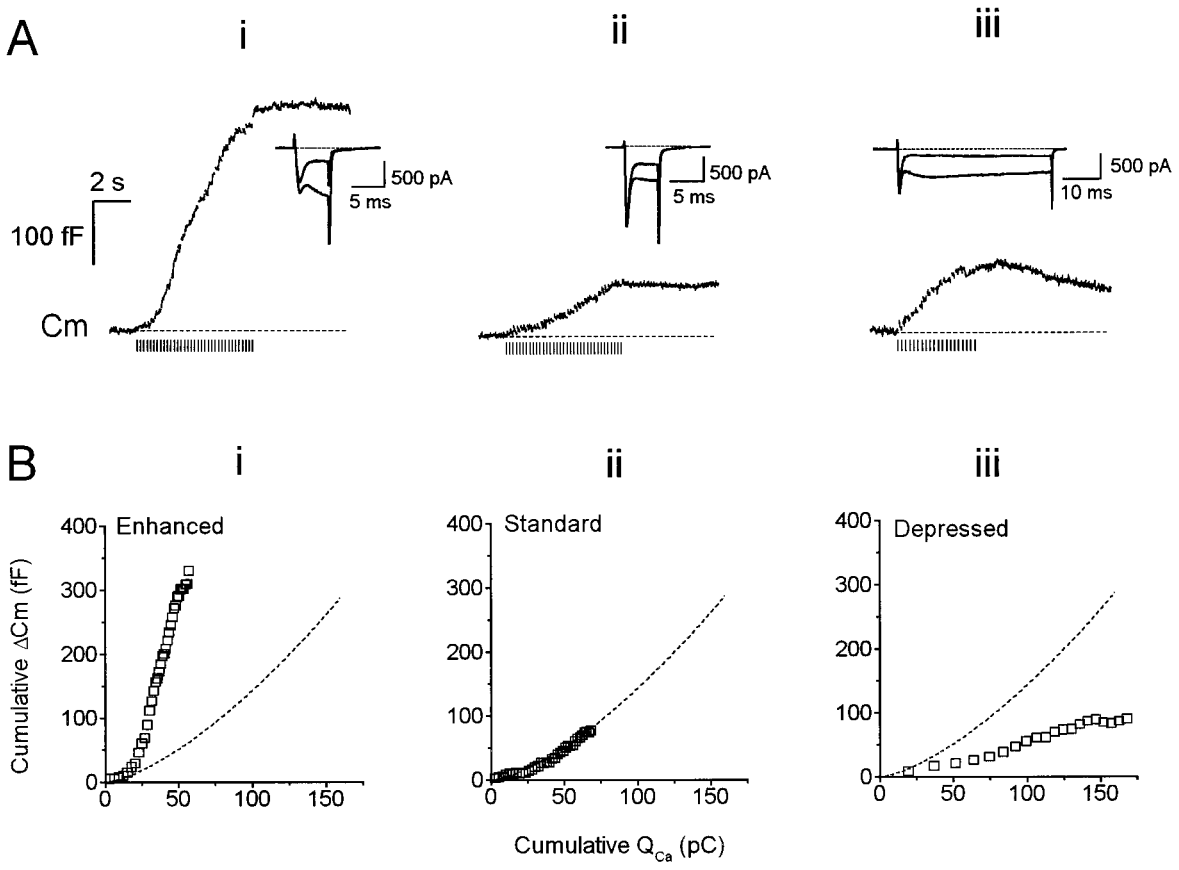

Figure 5. Examples of secretory behaviors observed during repetitive stimulation in untreated adrenal chromaffin cells. *A*, Cm changes evoked by stimulus trains of depolarizing pulses (−90 to +20 mV) applied at 200 msec intervals. The timings of the depolarizations are indicated by *gaps* and *vertical lines* beneath the traces. *Ai*, A large increase in Cm evoked by a train of 5 msec pulses. *Aii*, A smaller Cm increase evoked by a train of 5 msec depolarizations in a different cell. *Aiii*, In a third cell, a train of 40 msec depolarizations does not evoke substantially greater exocytosis than the train in *ii*. *Insets*, Inward currents evoked by the first and last depolarization of each stimulus train. *B*, Cm increases summed over the stimulus train, plotted as a function of cumulative integrated Ca^{2+} entry for the traces shown in *A*. In each panel the *dashed curve* is the standard single-pulse Ca^{2+}–exocytosis relationship (see Materials and Methods). *Bi*, The response abruptly shifts to an enhanced Ca^{2+}–exocytosis relationship after seven pulses. *Bii*, The response maintains the same relationship to integrated Ca^{2+} entry as the standard curve. Note a similar amount of total Ca^{2+} entry occurred in this and the cell in *i* (~60 pC). *Biii*, The response has a depressed Ca^{2+}–exocytosis relationship, compared with the standard curve. As a result, little exocytosis is evoked despite total Ca^{2+} entry >150 pC.

frequency. 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 χ^2 test). Finally, the shifts occurred although average Ca^{2+} 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 Ca^{2+} entry were compared. Thus, during repetitive stimulation a reduction in Ca^{2+} 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 Ca^{2+} currents and depolarization-evoked exocytosis in bovine adrenal chromaffin cells. Three IgGs inhibited only P/Q-type Ca^{2+} current, and two additionally affected N-type Ca^{2+} 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 $[Ca^{2+}]_i$ 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 Ca^{2+} entry and P/Q-type current amplitude roughly correlated with the size of CMAPs in the five LEMS patients,

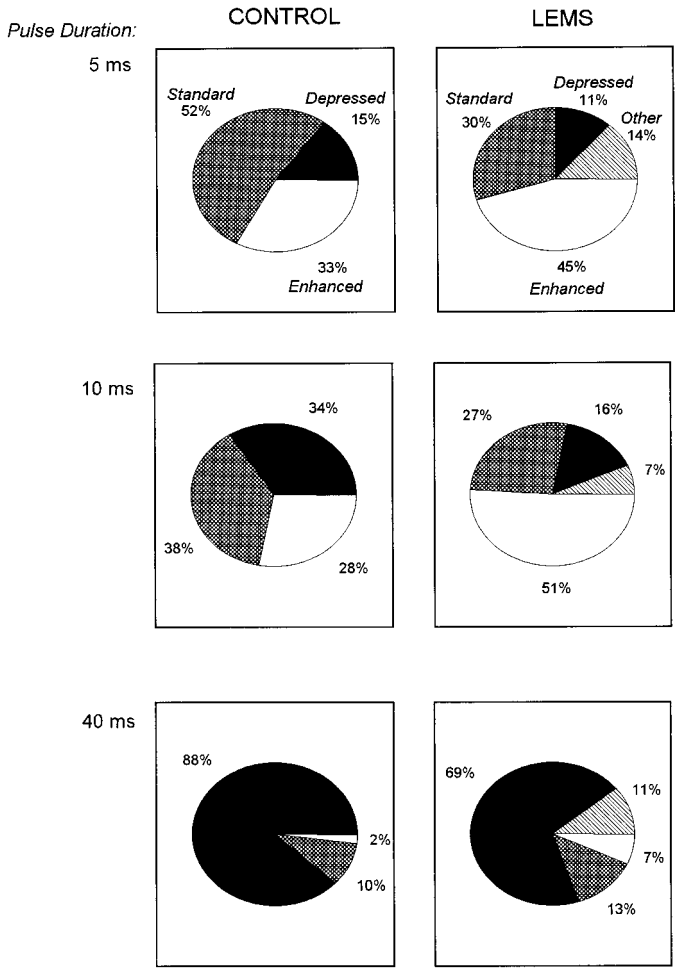

Figure 6. Enhancement during repetitive stimulation is more likely after treatment with LEMS IgGs. *Pie graphs* depict the distribution of response behaviors evoked by trains of depolarizing pulses. Control data are from Engisch et al. (1997, their Figs. 1–3), with 78 trains of 5 msec pulses, 42 trains of 10 msec pulses, and 93 trains of 40 msec pulses. LEMS data are from all cells treated with LEMS IgGs, with 44 trains of 5 msec pulses, 45 trains of 10 msec pulses, and 46 trains of 40 msec pulses. Only one train of any particular protocol was included per cell; multiple trains in the same cell were included if the protocols were different. Enhanced (*white sections*), standard (*cross-hatched sections*), and depressed (*black sections*) Ca^{2+}–exocytosis relationships were defined as being above, on, or below the standard single pulse relationship, respectively. A new category, *Other*, is shown as a *striped section* (also see Table 1).

whereas N-type current amplitude did not. These results suggest that the P/Q type Ca^{2+} channels inhibited by LEMS in chromaffin cells are similar to the P/Q-type Ca^{2+} channels mediating human neuromuscular transmission (Protti et al., 1996). Examining the effects of LEMS antibodies on Ca^{2+}-secretion coupling in chromaffin cells may give us insights into the underlying mechanism of the neuromuscular disease.

Small initial CMAPs in LEMS may be attributable to the reduction in Ca^{2+} channel number, but active zones are disorganized in the disease (Engel, 1991), and this or other effects of the antibodies could alter the Ca^{2+} dependence of release. At the neuromuscular junction (NMJ) the Ca^{2+} dependence of transmitter release must be inferred from the relationship between postsynaptic responses and extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Neurotransmitter release at the NMJ increases as the third or fourth power of $[Ca^{2+}]_o$, at very low $[Ca^{2+}]_o$ or high $[Mg^{2+}]_o$ (Dodge and Rahamimoff, 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 unchanged but shift the relationship to the right, because higher levels of $[Ca^{2+}]_o$ are required to evoke the same amount of release. The power of the relationship between $[Ca^{2+}]_o$ 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 $[Mg^{2+}]_o$ at the mouse NMJ after passive transfer of LEMS, a power dependence of 3.9 was observed, and the relationship between endplate potentials and $[Ca^{2+}]_o$ was indeed shifted to the right (Lang et al., 1987).

The dependence of transmitter release on Ca^{2+} influx, rather than $[Ca^{2+}]_o$, 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 ω-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 $[Ca^{2+}]_o$ and/or raising $[Mg^{2+}]_o$ (Del Castillo and Katz, 1954). Katz and Miledi (1968) proposed the residual Ca^{2+} hypothesis to explain activity-dependent facilitation of transmitter release: when $[Ca^{2+}]_o$ 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

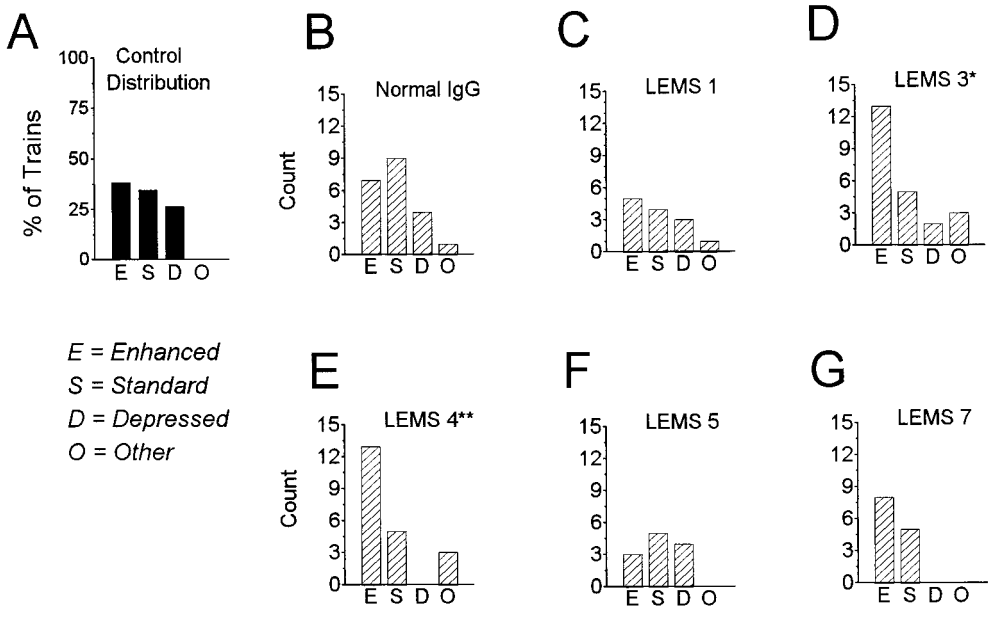

Figure 7. Distribution of secretory behaviors evoked by trains with small amounts of Ca^{2+} entry for the first pulse of the train. *A*, Control distribution, taken from Engisch et al. (1997). Low range of Ca^{2+} entry: $Q_{Ca} < 6.4$ pC. Percentage of trains in a large sample ($n = 106$) with the 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 χ^2 test.

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

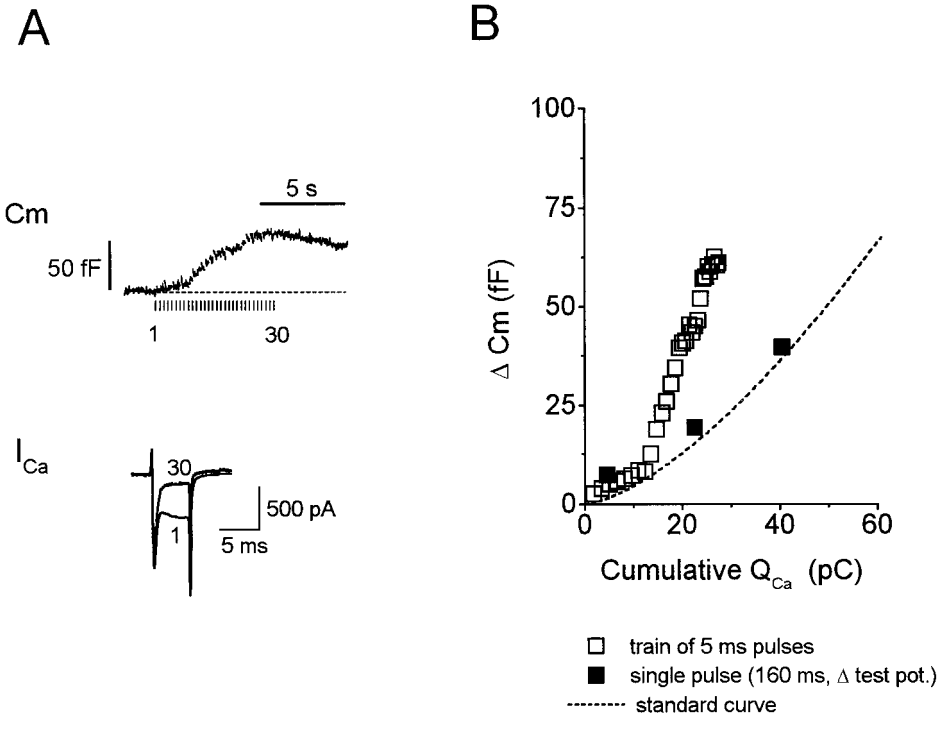

Figure 8. Comparison of single pulse responses with a train-evoked response in a LEMS 3-treated chromaffin cell. *A*, Membrane capacitance trace (*Cm*) recorded during repetitive stimulation with 5 msec depolarizing voltage steps (−90 to +20 mV) in a cell that had been treated for 24 hr with LEMS 3 IgG (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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} entry values. Second, LEMS 4 IgG increased enhancement without substantially inhibiting Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}–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.

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