IgG from Patients with Lambert-Eaton Syndrome Blocks Voltage-Dependent Calcium Channels

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Lambert-Eaton syndrome, an autoimmune disorder frequently associated with small-cell carcinoma of the lung, is characterized by impaired evoked release of acetylcholine from the motor nerve terminal. Immunoglobulin G (IgG) antibodies from patients with the syndrome, applied to bovine adrenal chromaffin cells, reduced the voltage-dependent calcium channel currents by about 40 percent. When calcium was administered directly into the cytoplasm, however, the IgG-treated cells exhibited normal exocytotic secretion, as assayed by membrane capacitance measurement. Measurement with the fluorescent calcium indicator fura-2 indicated that the IgG treatment reduced potassium-stimulated increase in free intracellular calcium concentration. The pathogenic IgG modified neither kinetics of calcium channel activation nor elementary channel activity, suggesting that a reduction in the number of functional calcium channels underlies the IgG-induced effect. Therefore, Lambert-Eaton syndrome IgG reacts with voltage-dependent calcium channels and blocks their function, a phenomenon that can account for the presynaptic impairment characteristic of this disorder.

IN THE LAMBERT-EATON MYASTHENIC syndrome (LES), the motor nerve terminals release an insufficient number of acetylcholine quanta in response to a nerve impulse (*1*). Patients with this acquired neuromuscular disorder frequently manifest cancer, particularly small-cell carcinoma of the lung (SCCL) (*1, 2*). Although the etiology of LES is uncertain (*3*), it is well established that the syndrome has an autoimmune pathogenesis (*4–6*). Mice injected with immunoglobulin G (IgG) molecules or plasma from patients with LES develop characteristic features of the human disease (*4, 5*). Such passive transfer studies demonstrate that an IgG antibody plays a specific pathogenic role in the presynaptic impairment. Presumably, the pathologically active IgG reacts with a presynaptic antigenic determinant, the function of which is perhaps essential for transmitter secretion. In support of this view, experiments with the mouse passive transfer model substantiate that the neuromuscular junctions treated with LES IgG exhibit characteristics consistent with the blockade of presynaptic calcium channels (*7–9*). Because of the inaccessibility of the motor nerve endings, however, these channels cannot be probed directly, thus hindering direct identification of the putative antigenic site recognized by LES IgG.

In this regard, the finding that LES IgG inhibits rat anterior pituitary hormone release and $^{45}Ca^{2+}$ uptake in vitro (*10*) provided an opportunity for direct examination of the antibody-antigen interaction. We applied IgG from patients with LES to another type of electrically excitable endocrine preparation—bovine adrenal chromaffin cells—and investigated some of the vital membrane processes by patch clamp techniques.

Immunoglobulin fractions containing IgG were prepared from the plasma of five patients with LES (*11*), three of whom had cancer (see Table 1), and six healthy control subjects. The antibody isolation was carried out with either the ammonium sulfate precipitation (*12*) or rivanol (*13*) method. Although these fractions contain plasma factors other than IgG, they will be henceforth referred to as LES or control IgG because the crude extracts transfer the disorder to mice as well as does purified IgG (*5*). In some experiments, the IgG used was purified by diethylaminoethyl-cellulose chromatography and confirmed to be pure by immunoelectrophoresis. After incubation with IgG for 2 or 24 hours, whole-cell and single Ca^{2+}-channel currents were measured according to the methods of Hamill *et al.* (*14*) with an external physiologic buffer free of IgG. Whole-cell currents were recorded either with or without simultaneous blockade of the transient Na^+ currents (I_{Na}). Membrane capacitance (C_m) measurement of exocytotic secretion and fura-2 measurement of free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were conducted by the methods of Neher, Marty, and Lindau (*15*) and Tsien and his co-workers (*16*), respectively.

The data based on whole-cell recordings of Ca^{2+} currents (I_{Ca}) from the cells incubated with IgG of five LES patients are given in Table 1. Figure 1A illustrates typical current traces obtained from two treated cells. In each experiment, the response of six to ten cells treated with one patient's IgG was compared with that obtained with IgG of a randomly selected control subject. None of the IgG from six control subjects significantly altered I_{Ca}, as revealed by comparison with the values found in normal untreated cells. In the cells incubated with control IgG for 2 and 24 hours, the amplitude of Ca^{2+} currents on average increased by 6 ± 4% (mean ± SEM; n = 67 cells) and 3 ± 3% (n = 101), respectively. However, I_{Ca} in the cells exposed to LES patients' IgG attained values consistently smaller than those observed with control IgG, with the reduction ranging from 35 to 57%. With antibodies of patients 2 and 3, this inhibitory effect was apparent after 30 minutes of incubation. Immunoglobulin G antibodies from patients with and without cancer were equally effective in blocking the Ca^{2+} channel currents. In general, the blocking effect of LES IgG became more pronounced after a 24-hour incubation. These effects were dose-dependent and significant. The antibodies appeared to act in an irreversible manner, because washing with IgG-free buffer for up

Table 1. Reduction in voltage-dependent calcium currents (I_{Ca}) in bovine adrenal chromaffin cells treated with LES IgG. I_{Ca} was recorded as in Fig. 1 from cells that had been continuously exposed to each patient's IgG for 2 or 24 hours. The amplitude of maximum inward I_{Ca} was normalized according to the cell size by dividing by the cell membrane capacitance C_m. The percent reduction was then calculated by comparison with the similarly normalized mean amplitude of I_{Ca} in control IgG-treated cells. The IgG dose in all preparations was 4 mg/ml except for that of patient 3 for which 1 mg/ml was used. Data are shown as means ± SEM; all reductions are significant, $P < 0.001$ by student's t test when compared to the control. The number of cells studied (n) and the range of percent reduction among different experiments are given in parentheses. RCC, renal cell carcinoma; SCCL, small-cell carcinoma of the lung.

LES patients	Associated cancer	Reduction in I_{Ca} (%)	
		2 hours	24 hours
1	RCC	35 ± 7 (n = 21; 21–65)	
2	SCCL	49 ± 7 (n = 28; 30–57)	57 ± 4 (n = 28; 51–63)
3	None	56 ± 6 (n = 25; 32–84)	
4	SCCL	40 ± 4 (n = 47; 18–62)	42 ± 3 (n = 50; 32–61)
5	None	38 ± 5 (n = 39; 20–58)	50 ± 4 (n = 32; 41–58)

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to 1 hour did not noticeably change I_{Ca}.

In the cells treated with LES IgG, the voltage dependence of whole-cell I_{Ca} was not altered (Fig. 1B). In both LES and control cells, the polarity of plateau Ca^{2+} currents reversed at close to +50 mV, while the maximum inward currents were produced at 0 mV. There was no apparent abnormality in the overall shape of the *I-V* curves, except for the proportionate decrease of I_{Ca} at all potential levels.

LES IgG appeared to block specifically the voltage-dependent Ca^{2+} channels. Unlike I_{Ca}, whole-cell Na^+ currents remained relatively unchanged from the control, with the exception of a significant decline ($P < 0.001$) in I_{Na} produced by IgG from patient 3 (*17*).

Purified LES IgG also reduced I_{Ca} but was less effective than the hitherto described Ig fractions. For example, purified IgG of patient 2, applied for 24 hours, caused only a 34 ± 5% reduction ($n = 31$), compared to 57 ± 4% found with IgG fraction isolated by the rivanol method. This implied that the pathogenic IgG might require certain humoral factors in producing more efficient channel blockade. In fact, we sometimes observed differences in the efficacy of IgG isolated by ammonium sulfate precipitation and that obtained by the rivanol method, especially when the incubation time was short. In the cells incubated for 2 hours, antibodies of patients 4 and 5 prepared by the former method reduced I_{Ca} to a significantly greater degree than did antibodies isolated by the latter. Thus, the former IgG samples might have contained extra humoral factors that presumably accelerated the pathological action of IgG (*18*). Consistent with this idea, when these samples were heat-inactivated (*19*), there was a sizable loss of their efficacy. I_{Ca} in the cells treated for 2 hours with unheated IgG extract fell by 53 ± 5% ($n = 29$) but fell only by 22 ± 6% ($n = 27$) when the heated extract was used.

Although the autoantibodies interfered with the function of voltage-dependent Ca^{2+} channels, they did not impair the cell's ability for exocytotic secretion. This was tested by measurements of cell membrane capacitance, which provided an electrical assay of exocytotic release (*15, 20*). We injected Ca^{2+} buffer through the recording patch pipette, thereby increasing the cytoplasmic Ca^{2+} concentration to about 1 μ*M*. The secretory activity consequent upon elevated $[Ca^{2+}]_i$ was then monitored by measuring C_m, which reflected the increase in membrane surface area as exocytotic vesicles were incorporated into the plasma membrane. Capacitance in both control and LES cells rose with a time course typical for the normal untreated cells (Fig. 2). In the cells treated for 24 hours with antibodies of patients 2, 4, and 5, C_m increased at a rate of 2.24 ± 0.29 pF/min (mean ± SEM, $n = 25$) during the first 2 minutes, being closely comparable to the control rate, 2.28 ± 0.28 pF/min ($n = 28$). Within about 10 minutes of Ca^{2+} exposure, membrane capacitance increased by a factor of 2.44 ± 0.69 ($n = 26$) relative to the initial C_m. This was virtually unchanged from the equivalent control value, 2.46 ± 0.80 ($n = 28$). Therefore, given access to cytoplasmic Ca^{2+}, exocytosis in the LES IgG-treated cells apparently proceeded normally, indicating that the autoantibodies do not act on the cellular mechanism directly responsible for exocytotic secretion. This is quite unlike the action of botulinum toxin type A and tetanus toxin (*20*), which prevent an increase in C_m after a similar high $[Ca^{2+}]_i$ stimulus. This observation further indicates the specificity of action of the autoantibodies.

Since voltage-sensitive Ca^{2+} channels undergo Ca^{2+}-dependent inactivation or "run-down" (*21, 22*), a reduction in I_{Ca} could occur as a consequence of raised $[Ca^{2+}]_i$. To

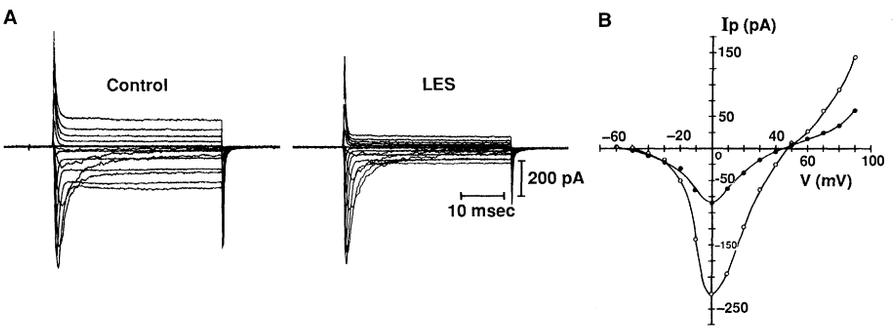

Fig. 1. (**A**) Whole-cell Ca^{2+} and Na^+ currents in the antibody-treated bovine chromaffin cells of adrenal medulla. The cells, prepared as in Fenwick *et al.* (*21*), were incubated at 37°C for 2 hours in the culture medium containing control or LES (patient 4) IgG (4 mg/ml). After incubation, the currents were measured at room temperature (22° to 24°C) with IgG-free physiologic bath solution. The cell culture medium was Medium 199 with Hanks salts supplemented with 10% fetal calf serum, bovine serum albumin, antibiotics, and glucose. All IgG samples were dialyzed against the cell culture medium before they were applied to the cells. The pipette filling solution contained 120 m*M* CsCl, 20 m*M* tetraethylammonium chloride, 11 m*M* EGTA-NaOH, 1 m*M* $CaCl_2$ (10 n*M* free Ca^{2+}), 2 m*M* $MgCl_2$, and 10 m*M* Hepes-NaOH, *p*H 7.2. The external bath solution had the following composition: 140 m*M* NaCl, 2.8 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, and 10 m*M* Hepes-NaOH, *p*H 7.2. Na^+ and Ca^{2+} currents were elicited by depolarizing voltage steps of 40-msec duration. With the membrane potential held at −80 mV (V_h), each cell was successively depolarized to 16 test potentials between −60 and +90 mV in 10-mV increments. Adequate diffusion of pipette solution into the cell was ensured by making the recordings at least 1 minute after the whole-cell configuration was achieved. The voltage-gated Ca^{2+} currents were measured as the plateau inward currents appearing at the end of 40 msec of depolarization. This criterion is reasonable because exposure of the cells to Ca^{2+}-free, 2 m*M* cobalt buffer resulted in a 99% inhibition of these plateau currents ($n = 24$ cells) (*21*). The two cells studied were of comparable size, as verified by the values of their membrane capacitance: 5.02 pF in the control and 5.49 pF in the LES cell. (**B**) Plots of plateau Ca^{2+} currents (I_p) appearing in (A) as a function of the depolarizing test potential *V* (open symbols, control; closed symbols, LES).

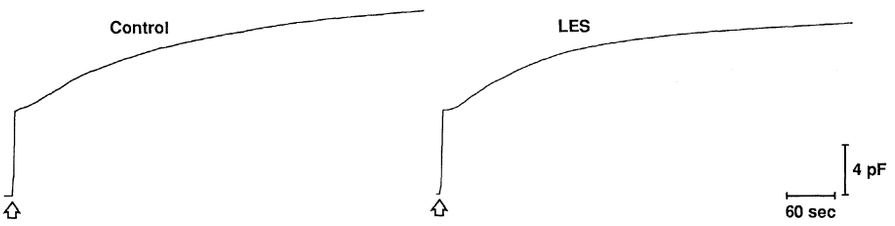

Fig. 2. Typical time course of the membrane capacitance changes occurring in response to 1 μ*M* cytoplasmic Ca^{2+}. These cells were incubated for 24 hours in the presence of control or LES (patient 5) IgG (4 mg/ml), as described in Fig. 1. The output of a two-phase lock-in amplifier, coupled with LIST EPC-7 patch clamp amplifier (List Electronic, Darmstadt, F.R.G.), was analyzed by a computer to derive signals representing changes in membrane capacitance (C_m) (*15*). The cells were excited by a 16-mV RMS sinusoidal voltage signal at a frequency of 800 Hz, while a holding potential of −80 mV was maintained throughout the measurement. Bath temperature was 30°C. The pipette solution, in which free Ca^{2+} concentration was buffered at about 1 μ*M*, was composed of 135 m*M* potassium glutamate, 20 m*M* NaCl, 0.5 m*M* adenosine triphosphate, 10 m*M* EGTA-NaOH, 9 m*M* $CaCl_2$, 4 m*M* $MgCl_2$, and 10 m*M* Hepes-NaOH, *p*H 7.2. The external bath solution was the same as that described in Fig. 1. The initial values of C_m, measured immediately after the establishment of the whole-cell configuration (as marked by the arrows), were 6.64 and 6.58 pF in control and LES cells, respectively. Approximately 10 minutes later, these values had risen to 14.45 and 13.42 pF.

examine this, we measured $[Ca^{2+}]_i$ in the cells loaded with the fluorescent Ca^{2+} indicator dye fura-2 (*16*). In 31 cells exposed for 24 hours to IgG of patients 2 and 4, the resting $[Ca^{2+}]_i$ was 127 ± 15 n*M*, marginally lower ($P > 0.2$) than the concentration found in 30 control IgG-treated cells, 140 ± 13 n*M*. This result thus argues against unspecific cell deterioration as the cause of the observed Ca^{2+} channel blockade. With external potassium concentration raised to 80 m*M*, $[Ca^{2+}]_i$ in LES cells increased to 204 ± 17 n*M*, a value significantly lower ($P < 0.001$) than the control, 310 ± 20 n*M*. This is consistent with what would be expected when the voltage-activated Ca^{2+} channels are blocked.

In order to examine the time course of Ca^{2+} channel activation after LES IgG treatment, we blocked Na^+ current with tetrodotoxin (TTX) (20 μg/ml), scaled the LES and control current traces evoked at 0 mV to an identical size, and superimposed them (Fig. 3A). In 15 LES cells incubated for 24 hours (with antibodies from patients 2 and 4), such a superimposition revealed no noticeable difference from the control. As demonstrated in the absence of TTX (see Fig. 1B), the whole-cell current-voltage relation remained unchanged from that of the control (Fig. 3B). Therefore, Ca^{2+} currents in LES and control cells were similar in both voltage dependence and kinetics, suggesting that the autoantibodies do not alter the temporal pattern of the Ca^{2+} channel opening.

Using cell-attached membrane patches, we examined the possibility that the autoantibodies act by modifying characteristics of elementary Ca^{2+} channel activity. The recordings were carried out on 20 m*M* K^+-depolarized cells, with the patch pipettes filled with isotonic Ba^{2+} solution (Fig. 3C). Mean unitary amplitude of the single Ca^{2+} channel currents was 0.87 ± 0.03 pA (n = 18 cells, treated for 24 hours with IgG of patients 2, 4, and 5), not significantly different from the control value of 0.89 ± 0.04 pA (n = 14). The amplitude distribution was unaffected, yielding a normal Gaussian pattern. Neither the temporal pattern of channel opening nor channel open time appeared to be altered. These findings can be interpreted as indicating that the pathogenic autoantibodies act by reducing the number of functional Ca^{2+} channels, rather than by a homogeneous modification of individual channel characteristics.

Our results provide direct evidence that in LES, with and without cancer, IgG antibodies block the voltage-dependent Ca^{2+} channels. Yet the pathological antibodies do not impair the capability of the cells for exocytotic secretion if Ca^{2+} channels are bypassed by direct application of Ca^{2+} through the recording pipette. Apparently, they inhibit the function of individual Ca^{2+} channels in all-or-nothing fashion, with no change in the kinetics of unblocked channels. Such a pathophysiologic mechanism of LES IgG can account for the deficiency in the impulse-evoked release of acetylcholine that characterizes this disorder. In chromaffin cells, Ca^{2+} entering through the channels leads to exocytotic secretion (*23*), as is the case at the motor nerve terminal (*24*), and the relation between I_{Ca} and secretion is expected to be highly nonlinear. For example, a 40% reduction in I_{Ca} caused by LES IgG could inhibit secretion by 80% or more if the secretion is related to the second or greater power of the Ca^{2+} channel current amplitude (*25*). In fact, such a degree of secretory inhibition is observed in human as well as murine passively transferred LES. The quantal content of the evoked end-plate potentials is reportedly reduced by about 90% in human patients (*1, 26*) and 75% in mice injected with LES IgG (*5, 8*). In view of the morphological finding that the syndrome depletes intramembrane particles associated with the presynaptic active zone (*9, 27*), our observation strengthens the concept that these particles may indeed be voltage-gated Ca^{2+} channels (*28*).

Of the three types of voltage-sensitive Ca^{2+} channels identified in neurons (*29*), L-type channels probably contribute to Ca^{2+} currents in adrenal medullary chromaffin cells (*21, 30*). Our finding thus indicates reactivity of LES IgG with L-type Ca^{2+} channels. Although the active zone Ca^{2+} channels responsible for presynaptic transmitter release are generally thought to be of N type, more recent studies postulate coexistence of N- and L-type channels and two characteristically different Ca^{2+} currents in the motor nerve terminal (*31*). Clinically, patients with the syndrome manifest a rather widespread adrenergic as well as cholinergic dysfunction of the autonomic nervous system (*32*). Therefore, the pathological action of LES IgG may not be confined solely to the neuromuscular synapse.

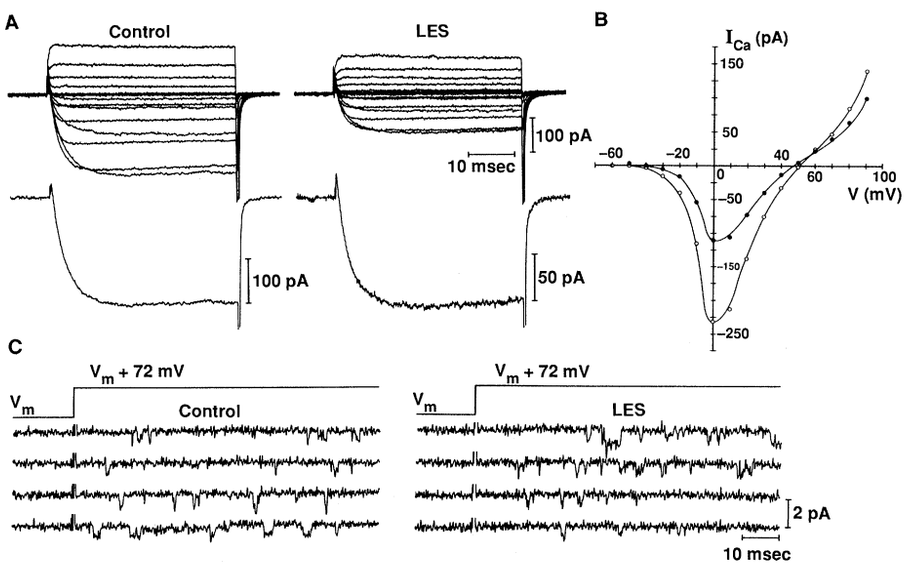

Fig. 3. Whole-cell and single-channel Ca^{2+} currents in the cells treated with control and LES IgG for 24 hours. (**A**) Upper traces: voltage-sensitive Ca^{2+} currents (I_{Ca}) recorded from the cells treated with control and LES (patient 2) IgG (4 mg/ml). These whole-cell currents were recorded as in Fig. 1 except that bath solution additionally contained TTX (20 μg/ml). Membrane capacitance of control and LES cells were, respectively, 5.61 and 5.76 pF. Lower traces: time course of Ca^{2+} currents at 0 mV, a test potential that elicited maximum inward currents. The currents taken from the upper ensemble were scaled for equal steady-state values to compare the time courses of Ca^{2+} channel activation. (**B**) Plots of I_{Ca} appearing in (A) as a function of the test potential V (open symbols, control; closed symbols, LES). (**C**) Single Ca^{2+} channel currents recorded in the cells incubated with control and LES (patient 4) IgG (4 mg/ml). Cell-attached patch clamp recordings were made with the pipettes filled with isotonic Ba^{2+} solution (96 m*M* $BaCl_2$ and 10 m*M* Hepes-NaOH, *p*H 7.2). The bath solution contained 128 m*M* NaCl, 20 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$ and 10 m*M* Hepes-NaOH, *p*H 7.2. Each panel shows four consecutive sweeps generated by 72-mV depolarizing voltage steps that were applied at 2-second intervals (filtered at 2 kHz and sampled at 100 μsec per point). V_m is the cell resting potential (approximately −50 mV for a 20 m*M* K^+-depolarized cell) minus pipette potential (+45 mV). Leakage and linear capacitive currents were subtracted. Remaining capacitive artifacts were truncated.

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18. Clearly, the putative humoral factor promoting the channel blockade is not specific for small-cell carcinoma of the lung. This view is supported by the observation that IgG fractions isolated by ammonium sulfate precipitation from three SCCL patients with no evidence of the syndrome failed to block I_{Ca}.
19. The samples were heated at 56°C for 30 minutes. It was assumed that this procedure inactivated residual complement proteins and heat-sensitive humoral factors, while not denaturing IgG.
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