Down-regulation of non-L-, non-N-type (Q-like) Ca^{2+} channels by Lambert-Eaton myasthenic syndrome (LEMS) antibodies in rat insulinoma RINm5F cells

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Abstract The action exerted on non-L-, non-N-type (Q-like) Ca^{2+} channels by immunoglobulins G (IgGs) obtained from two patients with Lambert-Eaton myasthenic syndrome (LEMS) was investigated in the rat insulinoma RINm5F cell line. LEMS IgGs reduced by 30–36% the whole-cell Ba^{2+} currents through Q-like Ca^{2+} channels at +10 mV without significantly modifying their voltage dependence and activation kinetics. Single- and multiple-channel recordings in cell-attached and outside-out patches of cells treated with LEMS IgGs showed no significant changes of the channel elementary properties but rather a decreased number of active channels per patch. This suggests that Q-like current depression by LEMS autoantibodies is mostly due to a down-regulation of functioning Ca^{2+} channels. In agreement with previous observations, LEMS IgGs also reduced by 20–33% the dihydropyridine-sensitive (L-type) Ba^{2+} current. The suggested down-regulation of Q-like channels by LEMS IgGs in RINm5F cells may have a functional correlation with the depressive action of LEMS autoantibodies on the P/Q-type Ca^{2+} channels controlling acetylcholine release from mammalian neuromuscular junctions.

Key words: Ca^{2+} channel; Lambert-Eaton myasthenic syndrome; IgG; Insulinoma; RINm5F cell

1. Introduction

Lambert-Eaton myasthenic syndrome (LEMS) is a paraneoplastic autoimmune disease, often associated to small cell lung carcinoma (SCLC), which is characterized by a widespread impairment of neurotransmitter release from nerve terminals [1,2]. Motor disturbances occurring in LEMS patients, such as muscle weakness and stretch reflex depression, have been attributed to a down-regulating action of LEMS autoantibodies on presynaptic Ca^{2+} channels causing a decrease in acetylcholine (ACh) release at the neuromuscular junction [3]. Thus, there is great interest in identifying the voltage-dependent Ca^{2+} channels targeted by LEMS IgGs as well as the mechanisms underlying the IgG–Ca^{2+} channel interaction. Early electrophysiological studies pointed to an inhibitory action of LEMS IgGs on L-type Ca^{2+} channels [4,5]. It has been shown, however, that L-type, as well as N-type channels, contribute marginally to the neurotransmitter release from motoneuron terminals of mammals [6,7]. ACh release in mammalian neuromuscular junctions is mainly controlled by a non-L-, non-N-type channel sensitive to ω-agatoxin IVA [8] and funnel-web spider toxin [9], i.e. by a P/Q-type channel [10]. Recent studies on different cellular preparations have also shown that LEMS IgGs exert an inhibitory action on several Ca^{2+} channel subtypes, including L-, N-, T-, and P-type [11–13]. While the action on N-type channels may account for the LEMS symptoms related to the altered catecholamine release from autonomic neurons [12,14], an effect on P/Q-type channels may be responsible for the motor function impairment in LEMS patients.

The possibility that LEMS IgGs may down-regulate P/Q-type channels prompted us to test the effect of these autoantibodies on a newly described non-L-, non-N Ca^{2+} channel ('Q-like') expressed in rat insulinoma RINm5F cells [15]. The channel possesses biophysical and pharmacological properties similar to those described for the P/Q-type channels of peripheral and central neurons and contributes to a large proportion of Ca^{2+} currents in these cells [16]. Here we show that IgGs purified from sera of two LEMS patients reduce the non-L, non-N Ca^{2+} currents of RINm5F cells by 31–36% without affecting their voltage dependence, activation kinetics and single-channel properties. This action may be related to the down-regulation of presynaptic P/Q-type channels controlling ACh release in mammalian neuromuscular junctions which represent one of the main targets of LEMS autoantibodies.

2. Materials and methods

Rat insulinoma RINm5F cells were cultured as previously reported [16,17]. Electrophysiological recordings were performed 4–6 days after plating. The standard internal solution was (mM): 110 CsCl, 10 TEACl, 2 $MgCl_2$, 10 EGTA, 10 glucose, 10 HEPES (pH 7.3 with CsOH) and 0.1 μM TTX. 4 mM ATP and 0.2 mM cAMP were added to the internal standard solution to slow down the run-down of Ba^{2+} currents. The external recording solution was (mM): 120 NaCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES and 0.3 μM TTX to block Na^+ currents (pH 7.3 with NaOH). Nifedipine (Bayer AG) was used at 5 μM final concentration from a 1 mM stock dissolved in ethanol (95%) and stored in the dark at 4°C. ω-CTx-GVIA was dissolved in distilled water and kept in stock aliquots at −20°C until usage at final concentration of 3.2 μM. To maximize the irreversible blocking effect of ω-CTx-GVIA, RINm5F cells were soaked for 15 min in standard Tyrode's solution (2 mM [Ca^{2+}]) containing the toxin. The perfusion system for solution exchange consisted of a multi-barrelled pipette connected to five syringes through teflon tubes (flow rate 1–2 ml/min).

Before electrophysiological recordings, the cells were incubated overnight in culture medium with IgGs (1–2 mg/ml) obtained from either healthy subjects (control) or LEMS patients (H and D). IgGs were purified from sera by ammonium sulfate precipitation, then dialysed against phosphate buffer saline, lyophilized and kept frozen in stock solutions (20 mg/ml) until use (see [2,14]).

Electrophysiological recordings were performed with the patch-

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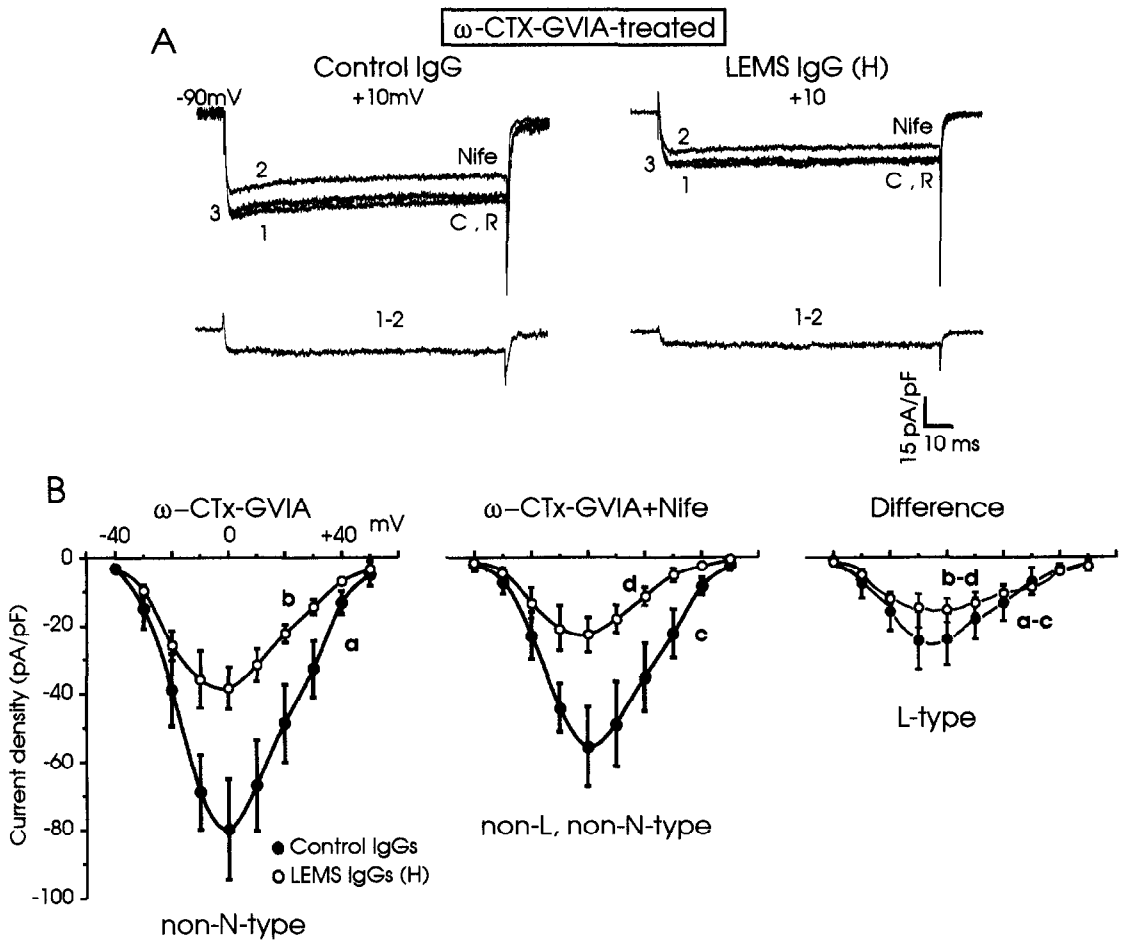

Fig. 1. Inhibition of HVA Ca^{2+} channels induced by LEMS IgGs (H) in RINm5F cells pre-treated with 3.2 μM ω-CTx-GVIA. After overnight incubation with either control or LEMS IgGs (2 mg/ml), the whole-cell Ba^{2+} currents were recorded before (C), during addition of 5 μM nifedipine (Nife) and after wash-out (R). Current amplitudes were normalized to cell capacitance. A: Representative Ba^{2+} current traces recorded at +10 mV from −90 mV holding potential showing the marked reduction of current size induced by LEMS IgGs (H). The L-type component (lower traces), obtained by subtracting the Nife trace (2) from the C trace (1), is also reduced by LEMS IgGs. Note that the nifedipine block of control cells was completely reversible in 30–45 s and was smaller than previously reported (25% vs. 55%, $n=30$) [15,17], indicating a lower density of L-type channels expression in the RINm5F cells used for these experiments. B: I/V relationships of total (non-N-type), Q-like (non-L-, non-N-type) and DHP-sensitive (L-type) Ba^{2+} currents for control- (filled circles) and LEMS IgGs-treated cells (empty circles). Data points were collected from 8 cells treated with control IgGs and 8 cells treated with LEMS IgGs. Notice that average current amplitudes and percentage of LEMS IgGs inhibition differ from those derived from a larger number of cells ($n=28$), due very likely to some variability of action of LEMS IgGs on different groups of cells. The I/V curves for the L-type channel were obtained by subtracting curve a and c and curve b and d, as indicated. Cells were pretreated with 3.2 μM ω-CTx-GVIA and exposed to 5 μM nifedipine as detailed in panel A.

clamp technique in whole-cell and cell-attached configurations [18] using the List EPC-7 amplifier (Darmstadt, Germany). Patch pipettes were pulled from borosilicate glass with a final tip resistance of 4–6 MΩ for both configurations. For single channel experiments, electrode tips were coated with Sylgard 184 (Dow Corning Corp., Midland, MI, USA) to lower the pipette capacitance. Macroscopic currents were acquired at 10–20 kHz (pClamp 5.0; Axon Instruments) and filtered at 5 kHz with an 8-pole low-pass filter. Single-channel recordings were sampled at 5–10 kHz and filtered at 2 kHz. The stimulation protocol consisted of voltage steps from −90 mV holding potential to voltages ranging from −40 to +50 mV. Most of the data refer to +10 mV test pulses. In whole-cell experiments, capacitive transient and leakage current compensation was performed both on line by the clamp-amplifier settings and off line by either subtracting Cd^{2+}-insensitive currents (200 μM Cd^{2+}) or using the P/4 technique with hyperpolarizing pulses from −90 mV. Current densities (pA/pF) were calculated by dividing the current amplitude by the C_{slow} setting required to minimize the cell capacity transient. To improve the clamp response, the electrode series resistance was compensated by 30–45%. In cell-attached and outside-out patch recordings, capacitive and leak currents were corrected by subtracting an average of 'null' sweeps. Data analysis was done using AutesP software (Garching Innovation, Munich, Germany) [16]. Data are expressed as mean ± S.E.M. for n = number of cells. Student's t-test was used for statistical evaluation.

3. Results

Of the three high-voltage-activated (HVA) Ca^{2+}-channel subtypes identified in RINm5F cells (N-, L-type and Q-like) [15], the N-type contributes only a minor proportion of total Ba^{2+} currents (10–15%) [17,19]. Its presence complicated the separation of the two other HVA channel currents; therefore, all the effects of LEMS IgGs described here were tested on RINm5F cells pre-treated with 3.2 μM ω-CTx-GVIA (15 min in 2 mM Ca^{2+}) which abolished N-type channel activity.

We first studied the effects of 2 mg/ml control and LEMS IgGs from patient H (IgGs (H)) on the total Ba^{2+} currents in ω-CTx-GVIA-treated cells (Fig. 1A). Ba^{2+} current amplitude at +10 mV in cells treated with control IgGs (45.4 ± 4.1 pA/pF, $n=28$) was significantly larger than that of cells treated

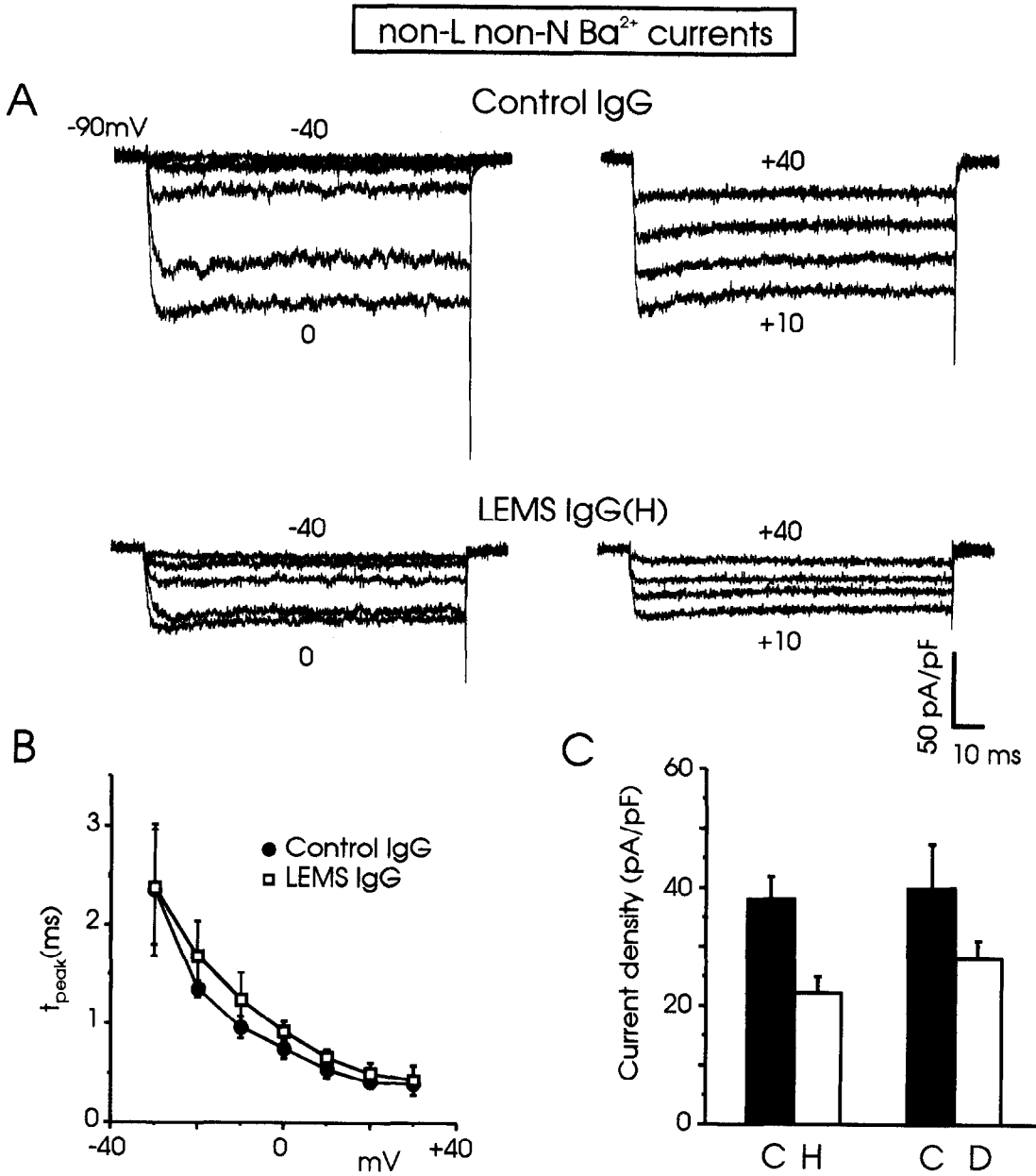

Fig. 2. Effects of control and LEMS IgGs on non-N, non-L type Ca^{2+} channels in RINm5F cells chronically treated with 3.2 μM ω-CTx-GVIA and 5 μM nifedipine. A: Whole-cell Ba^{2+} currents recorded at potentials from −40 to +40 mV with step increments of 10 mV in cells treated with either control IgGs (top traces) or LEMS IgGs (bottom traces). B: Time-to-peak (t_{peak}) vs. voltage for non-L-, non-N-type Ba^{2+} currents recorded in control- (dots, $n=8$) and LEMS IgGs (H)-treated cells (squares, $n=5$). C: Comparison of the inhibitory effects induced by IgGs purified from sera of two different LEMS patients (H and D). Current amplitudes measured at +10 mV show comparable reductions (36.2% for patient H, $n=28$, $P<0.01$ and 30.7% for patient D, $n=18$, $P<0.05$).

with LEMS IgGs (31.1 ± 3.6 pA/pF, $n=29$), indicating a 32% current decrease by LEMS autoantibodies ($P<0.02$). In order to evaluate the inhibitory effects of LEMS IgGs (H) on L-type and Q-like channels separately we then measured the size of the current remaining during addition of 5 μM nifedipine (Q-like) and the size of the current blocked by the DHP (L-type) in cells treated with LEMS and control IgGs. The residual Q-like current recorded at +10 mV decreased from 35.2 ± 3.6 pA/pF ($n=28$) in control IgGs to 22.5 ± 2.6 pA/pF ($n=28$) in LEMS IgGs (H)-treated cells, indicating a current reduction of 36.2% ($P<0.01$). L-type currents were also depressed, but significantly less. The mean block of L-type currents was 20.5% ($n=25$). The stronger action of LEMS IgGs on Q-like channels than L-type channels was evident on a wide range of membrane potentials (−40 to +40 mV, Fig. 1B). The I/V curves from 8 cells with significantly larger control currents (67.1 ± 13.0 pA/pF at +10 mV) were compared to those of 8 other cells treated with LEMS IgGs (H). Q-like channels were inhibited by 40% at −20 mV and by 68% at +20 mV (middle panel in Fig. 1B) while the decrease of L-type channels was significantly lower: 23% at −20 mV and 18% at +20 mV (right panel). The stronger inhibition of non-L-, non-N-type channels by LEMS IgGs at positive potentials was unexpected and could be due to the existence of non-L-, non-N-type

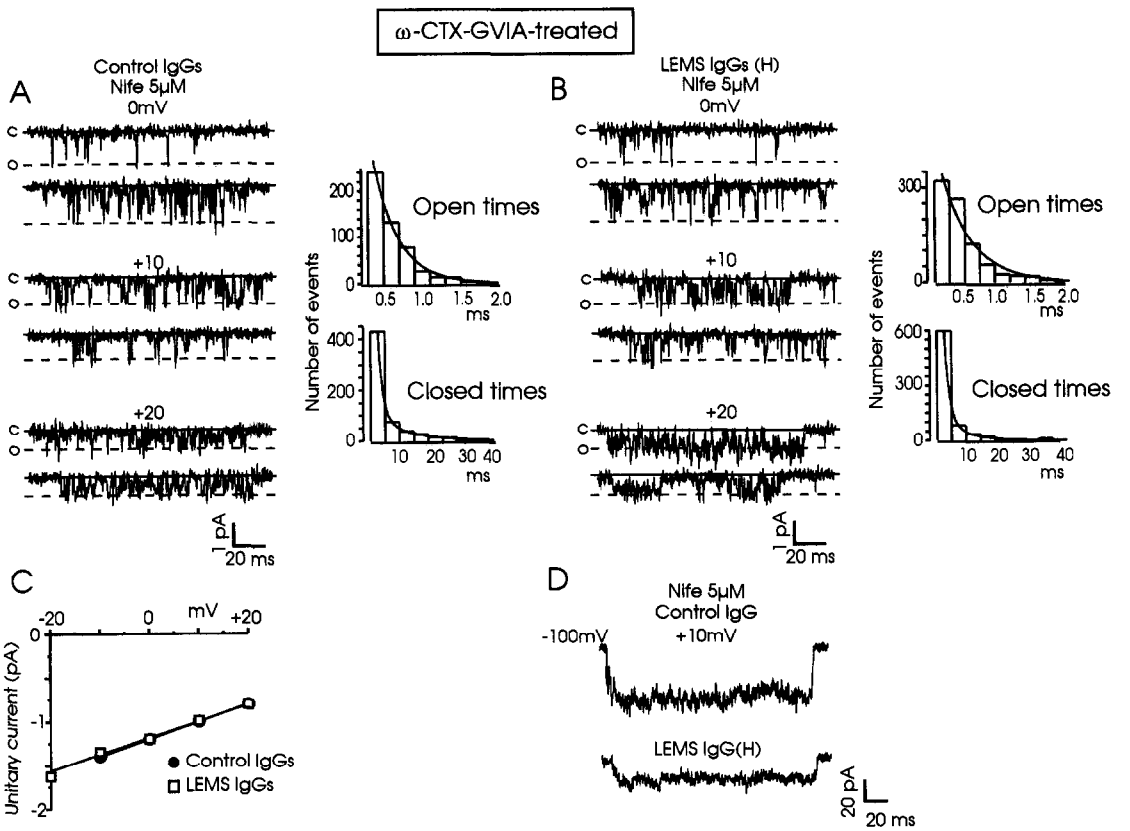

Fig. 3. The kinetic parameters of single non-N-, non-L-type channel are not significantly modified by LEMS IgGs. Unitary Ba^{2+} currents were recorded in cell-attached patches of RINm5F cells chronically treated with ω-CTx-GVIA and nifedipine after incubation with either control IgGs (A) or LEMS IgGs (B). Upper diagrams are open time distributions at +10 mV. The curves are single exponential functions with τ_o 0.39 ms (A) and 0.42 ms (B). Lower diagrams are closed time distributions at +10 mV fitted with double exponential functions with τ_{c1} 1.6 ms, τ_{c2} 9.6 ms (A) and τ_{c1} 1.6, τ_{c2} 11 ms (B). C: Unitary current amplitude versus voltage for control- (dots) and LEMS IgGs-treated cells (squares). Same conditions as panels A and B. The regression lines through data points have a slope conductance of 19.2 and 20.0 pS, respectively. D: Ensemble Ba^{2+} currents obtained by adding 45 sweeps from 19 outside-out patches of either control- (top trace) or LEMS IgGs-treated cells (bottom trace) containing multiple non-N-, non-L-type channels.

channel subpopulations with different sensitivity to LEMS IgGs: one more sensitive and activating at more positive potentials and one resistant to LEMS IgGs activating at slightly more negative potentials. This point was not further investigated in this study. Notice, however, that the percentage of inhibition of Q-like channels derived from Fig. 1B (63.3% at +10 mV, $n = 8$ cells) is much larger than that derived from 28 cells at the same potential (36.2%), suggesting a variability of LEMS IgGs action among groups of cells.

LEMS IgGs (H) had little effect on the activation-inactivation kinetics of Q-like channels. The voltage dependence and absolute values of the time-to-peak (t_{peak}) at potentials between −30 and +30 mV did not significantly differ in control and LEMS IgG-treated cells (Fig. 2A). In both groups of cells, t_{peak} decreased in a sharply voltage-dependent manner from 2.8 ms at −30 mV to 0.3 ms at +30 mV. At +10 mV, t_{peak} was 0.54 ± 0.09 ms in control IgG- and 0.66 ± 0.08 ms in LEMS IgG-treated cells. Similar effects were observed while testing the action of LEMS IgGs of a second patient (D). Q-like currents at +10 mV were significantly reduced by LEMS IgGs (D) but by a minor percentage in comparison to the IgGs of patient H (30.7% vs. 36.2%, Fig. 2C). LEMS IgGs (D) also reduced Ba^{2+} currents through L-type channels by 33.1%, i.e. by a larger degree than LEMS IgGs (H) (20.5% reduction) but by a percentage comparable to that induced by the same autoantibodies on the L-type channel of human neuroblastoma IMR32 cells (30.0% reduction [12]).

To verify whether LEMS IgGs affected either the number or the elementary properties of functioning channels, the activity of Q-like channels was studied in cell-attached and outside-out patches. Cells were pre-treated with ω-CTx-GVIA and exposed to saturating doses of nifedipine. Single Q-like channel activity recorded in cell-attached patches of cells treated either with control IgGs (Fig. 3A) or with LEMS IgGs(H) (Fig. 3B) showed strong similarities. In both cellular populations, non-L-, non-N-type channels started to activate positive to 0 mV from −90 mV holding potential. The channel open probability (p_o) at +10 mV was not significantly different (9.9 ± 1.2% in control cells and 12.5 ± 1.6% in LEMS IgGs (H), $P < 0.05$) and the same was true for the single channel conductance (γ) estimated between −20 and +20 mV. γ was 19.2 pS in control patches and 20 pS in LEMS IgGs (H)-treated patches (Fig. 3C). The open time histogram distribution at +10 mV was fitted by a single exponential function with a mean open time (τ_o) of 0.39 and 0.42 ms while the closed time histogram distribution was fitted by two exponentials with corresponding values: 1.6 and 9.6 ms for cells treated with control IgGs and 1.6 and 11 ms for LEMS IgGs (H)-treated cells. These results suggest that LEMS IgGs do not interfere with channel p_o but rather down-regulate the number

of functioning channels. Direct evidence for this comes from the observation that the probability of finding active channels in LEMS IgGs (H)-treated patches was significantly smaller compared to control IgGs-treated patches (only in 14 out of 31 patches). On the average, control patches always contained a higher number of active channels than LEMS IgGs (H)-treated patches. This was particularly evident in outside-out patches that usually contained multiple channels. In 16 out of 17 control outside-out patches we could observe an average activity of more than 3 channels, while in LEMS IgGs (H)-treated patches channel activity was limited to 1 or 2 channels and detected only in 9 out of 17 patches. The reconstituted currents obtained by averaging 45 sweeps from an equal number of control and LEMS IgGs (H)-treated patches had markedly different size but similar activation-inactivation kinetics (Fig. 3D). The averaged currents in LEMS IgGs (H)-treated cells were 57% smaller than control patches, indicating a reduction in the number of active channels probably due to a down-regulation of Q-like channels as already reported for other Ca^{2+} channels [4,12].

4. Discussion

The presence of a significant fraction of non-L, non-N-type Ca^{2+} channels in RINm5F cells, with biophysical and pharmacological properties close to the Q-type channel, makes this cell line a suitable model to investigate the inhibitory action of LEMS IgGs on the P/Q-type channels controlling the presynaptic Ca^{2+} entry in mammalian neuromuscular junctions [9]. ACh release in motor nerve terminals is insensitive to N- and L-type channel blockers [6,7] while it is reversibly abolished by nanomolar concentrations of ω-Aga-IVA and by 10^4-fold higher concentrations of ω-CTx-MVIIC [8]. Thus, the pharmacological profile of the non-L, non-N-type channel controlling neurotransmitter release in mammalian motoneurons is close, although not identical, to the P-type channel of central neurons [10]. The P-type channel of cerebellar Purkinje neurons is irreversibly blocked by nanomolar concentrations of ω-Aga-IVA and micromolar amounts of ω-CTx-MVIIC [20]. In this respect, the non-N, non-L-type channel of RINm5F cells resembles more the P-type channel of motoneuron terminals than that of central neurons: it is reversibly blocked by nanomolar concentrations of ω-Aga-IVA and irreversibly abolished by micromolar concentrations of ω-CTx-MVIIC [15]. Channels containing class A α_1 subunits may form a broad family of non-L-, non-N type Ca^{2+} channels with different sensitivity to ω-Aga-IVA and ω-CTx-MVIIC but close structural and biophysical properties. The effects observed on non-L, non-N-type channels of RINm5F cells may be suggestive of LEMS IgGs action on similar class A Ca^{2+} channels expressed in motoneuron terminals.

Our data show that LEMS IgGs inhibit the Ba^{2+} currents through the non-L, non-N-type channels of RINm5F cells by down-regulating the number of functioning channels. Macroscopic Ba^{2+} currents were reduced by 31–36% with no changes of their activation kinetics and single channel current properties. The finding that LEMS IgGs down-regulate P/Q-type channels is in agreement with results of immunoprecipitation studies showing the presence of anti-P/Q-type Ca^{2+} channel autoantibodies in sera of 62 out of 65 LEMS patients [21,22] and with a voltage-clamp study showing that LEMS IgG reduces the P-type channel current in SCLC cells [13]. These cells had been previously shown to express mRNA for P-type Ca^{2+} channels [23,24] and to possess a Ca^{2+} conductance insensitive to DHPs and ω-CTx-GVIA but depressed by nanomolar concentrations of ω-Aga-IVA [24]. The experimental evidence that SCLC cells express non-L-, non-N-type channels, the presence of IgGs against these channels in most LEMS sera and the finding that class A channels control ACh release from mammalian motoneurons, suggest that the marked down-regulation of non-L-, non-N-type channels by LEMS IgGs reported here may be functionally correlated to the impairment of motor function observed in LEMS patients.

We have recently shown that LEMS IgGs also down-regulate N-, L- and T-type Ca^{2+} channels in human neuroblastoma IMR32 cells, reducing the corresponding Ba^{2+} currents up to 55%, 49% and 48%, respectively [12]. These data, together with the present finding of an inhibitory action on Q-like Ca^{2+} channels and the depression of Na^+ currents reported in SCLC cells [25], suggest that LEMS IgGs exert a broad action on different voltage-dependent channels. The question then arises if there exists any specificity in LEMS IgGs action and what might be the origin of its variability on the different Ca^{2+} channel subtypes. A possible explanation for the broad action of LEMS IgGs is that different channels may share common antigenic sites to which the autoantibodies are directed. Ca^{2+} and Na^+ channel α_1 subunits in fact possess regions of considerable amino acid sequence homology [26] which may result in some structural and antigenic similarities among these channels [21]. On the other hand, SCLC cells, which are considered responsible for the autoimmune response in LEMS patients, express a variety of Ca^{2+} channels: L-, N- and P/Q-types [23,24] as well as Na^+ and K^+ channels [27]. It seems reasonable, therefore, that each one of these channel proteins may represent an independent antigenic stimulus for the production of specific autoantibodies. A different immune response occurring in each patient might then account for the variable proportions of Ca^{2+} channel subtypes targeted by LEMS autoantibodies. This might also explain the large variability in the severity of the disease as well as the degree of neurological complications observed in these patients. The inhibitory action exerted by LEMS IgGs on N-type [12,14] and P-type channels ([13,21] and present data) may account for the impairment of neurotransmitter release from autonomic and motoneuron terminals, respectively, thus explaining the most frequently observed symptoms in LEMS patients. Whether inhibition on L- and T-type channels has any correlation with the pathogenesis of LEMS symptoms remains still to be elucidated.

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