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Immunoassays Fail to Detect Antibodies Against Neuronal Calcium Channels in Amyotrophic Lateral Sclerosis Serum

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Recent studies suggested that autoantibodies that bind to voltage-dependent calcium channels and activate calcium entry may play a role in the progressive degeneration of motoneurons in sporadic amyotrophic lateral sclerosis. Immunoassays were performed to assess autoantibody titer in patients with amyotrophic lateral sclerosis or Lambert-Eaton myasthenic syndrome, a disease in which the presence of anti–calcium channel antibodies is well documented. Based on immunoprecipitation assays for antibodies against N-type calcium channels, only 8% (2/25) of amyotrophic lateral sclerosis patients had marginally positive titers, whereas 58% (18/31) of patients with Lambert-Eaton myasthenic syndrome had positive titers. Enzyme-linked immunosorbent assays with purified neuronal N-type calcium channels revealed immunoreactivity in 2 of 25 amyotrophic lateral sclerosis sera and 12 of 31 Lambert-Eaton myasthenic syndrome sera, which is not compatible with suggestions that enzyme-linked immunosorbent assay is a more sensitive technique for the detection of autoantibodies in amyotrophic lateral sclerosis. Furthermore, based on immunoprecipitation assays, amyotrophic lateral sclerosis sera were totally negative for antibodies against L-type calcium channels from skeletal muscle or brain. These data do not support the hypothesis that an autoimmune response against calcium channels plays a primary role in amyotrophic lateral sclerosis.

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Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease primarily affecting motor neurons in the spinal cord, brainstem, and cerebral motor cortex. Although the etiology and pathogenesis of sporadic ALS remain unknown, the possibility that autoimmunity could play a primary role has been examined for several years [1].

A series of reports by Appel and colleagues (reviewed in [2]) provides evidence that autoantibodies that activate voltage-gated calcium channels may be involved in initiating neurotoxicity. Immunoglobulins from ALS patients increase spontaneous quantal acetylcholine release from mouse motor neuron terminals, suggesting that calcium influx through the voltage-dependent calcium channels coupled to exocytosis may be enhanced [3]. At least five distinct classes of calcium channels are expressed in neurons [4]. P/Q-Type channels are thought to play a major role in controlling transmission at the mammalian neuromuscular junction [5], although N-type channels may also be involved [6]. Voltage clamp recording has shown that ALS IgG can increase channel activity in cerebellar Purkinje neurons where P-type currents predominate [7], in *Xenopus* oocytes heterologously expressing N-type currents [8], and in a hybrid motor neuron cell line [9]. Results of further experiments in the motor neuron cell line were consistent with the hypothesis that antibody-mediated channel activation triggers neurotoxicity. Incubation with ALS IgG reduced cell survival and an evaluation of the protective effects of selective channel antagonists indicated that N- and P/Q-type, but not L-type channels, mediated toxicity [10].

Immunoassay of an anti–calcium channel specificity in ALS sera would provide crucial support for antibody-mediated channel activation. Appel and colleagues [11] reported that 75% of patients with sporadic ALS produce antibodies that react with L-type calcium channels purified from rabbit skeletal muscle in enzyme-linked immunosorbent assays (ELISAs). However the observations outlined in the preceding paragraph predict that autoantibodies also react with N- or P/Q-type calcium channels. Calcium channels

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solubilized from neuronal membrane preparations can be specifically labeled with radioactive antagonists. Assays based on the capture of channel-radioligand complexes have been used to quantify autoantibodies in Lambert-Eaton myasthenic syndrome (LEMS) [12–17], a disease in which a primary role for anti–calcium channel autoantibodies is more clearly established (reviewed in [18]).

In this report we describe the use of immunoprecipitation assays and ELISA with purified N-type calcium channels from rat brain [19] to detect anti–neuronal calcium channel antibodies. Although both methods revealed autoantibodies in LEMS sera, immunoreactivity was not consistently detected in sera from patients with ALS.

Materials and Methods

Patients

Twenty-five patients had a diagnosis of probable or definite ALS as outlined by the El Escorial World Federation of Neurology criteria for the diagnosis of ALS [20]. The 31 patients given a diagnosis of LEMS included 15 patients with no detected neoplasm, 15 patients with small-cell lung carcinoma, and 1 patient with epidermoid carcinoma.

Immunoprecipitation Assays

ω Conotoxin GVIA (ωGVIA) was purchased from the Peptide Institute and sodium^{125} iodide ($Na^{125}I$) (2,200 Ci/mmol) from New England Nuclear. ωGVIA was radio-iodinated by the lactoperoxidase method, and mono[^{125}I]-iodo ωGVIA was purified by reverse-phase high-performance liquid chromatography (HPLC) on an analytical C18 column. A crude synaptosome (P2) fraction from rat brain (0.3 mg of protein/ml) was incubated with 0.2 nM [^{125}I]-ωGVIA in 25 mM Tris and 0.15 M sodium chloride (NaCl), adjusted to pH 7.4 with hydrochloric acid (HCl) (buffer A), overnight at 4°C. Membranes were pelleted and then resuspended and solubilized in buffer A containing 1.5% (wt/wt) Triton X-100. Insoluble material was removed by ultracentrifugation. Aliquots of the supernatant containing 6 to 8 fmol of [^{125}I]-ωGVIA–labeled N-type calcium channels were incubated with 5 μl of serum for 4 hours at 4°C in a final volume of 0.1 ml, completed with 20 mM sodium phosphate buffer and 0.1% Triton X-100, pH 7.4 (buffer B). One-tenth of a milliliter of protein A–cross-linked agarose beads (Sepharose Fast Flow) equilibrated in buffer B was added, followed by rotary mixing for 1 hour at 4°C. The beads were then recovered by centrifugation, washed by resuspension in 1 ml of buffer A containing 0.1% Triton X-100, and immune complexes were measured by gamma counting.

[^{3}H]Isradipine (PN200-110) (70–85 Ci/mmol) from Amersham was used to label L-type calcium channels in rat brain synaptosomal membranes or skeletal muscle microsomes. Solubilization, partial purification on wheat germ agglutinin (WGA)–cross-linked 4% agarose beads (Sepharose 4BCL) and immunoprecipitation assays were performed as previously described [16].

Enzyme-Linked Immunosorbent Assay

N-type calcium channels were purified from 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS) extracts of rat brain synaptosomes by sequential chromatography on chelating Sepharose Fast Flow (Sigma) and heparin-cross-linked 4% agarose beads (Ultrogel) (Sepracor) according to the method of Leveque and colleagues [19]. Purified proteins were dialyzed against 0.1 M sodium bicarbonate, pH 9.5, and then adsorbed onto microtitration plates (Costar) by adding 0.1 ml containing 0.3 μg of protein per well and incubating overnight at 4°C. Plates were washed with buffer A, blocked with 5% low-fat-milk powder and 0.05% Tween 20 in buffer A for 2 hours at 37°C, and then incubated with sera diluted tenfold in the blocking buffer, for 2 hours at 37°C. Plates were washed four times with 0.05% Tween 20 in buffer A, incubated with anti-human polyvalent immunoglobulins–alkaline phosphatase conjugate (Sigma) diluted 4,500-fold for 1 hour at 37°C, and again washed four times with 0.05% Tween 20 in buffer A. The optical density at 405 nm was measured 1 hour after the addition of 0.5 mg/ml of *p*-nitrophenyl phosphate in 10 mM diethanolamine containing 0.5 mM magnesium chloride.

Results

A membrane preparation containing rat brain nerve terminals was incubated with 0.2 nM ^{125}I-ωGVIA, a specific ligand for N-type calcium channels, and ligand-receptor complexes were extracted with Triton X-100. Sera were incubated with Triton X-100 extracts containing the prelabeled N-type channels, and immune complexes were trapped on protein A–Sepharose beads. Gamma counting provides a measurement of the recovery of calcium channels and thus of anti–N-type calcium channel autoantibody titer. Autoantibodies were not consistently detected by this method in sera from a group of ALS patients (Fig 1). Only 2 ALS patients (8%) from a group of 25 displayed titers marginally above (260 and 190 pmol) the limit of the normal range (160 pmol) defined as the mean titer + 3 standard deviations [SDs] of a group of 14 normal subjects. In contrast, sera from 18 LEMS patients (58%) from a group of 31 gave a positive response.

In view of reports that ALS IgG reacts with L-type calcium channels from skeletal muscle [11], immunoprecipitation assays were also performed using [^{3}H]PN200-110, a radioligand that binds specifically to L-type calcium channels. [^{3}H]PN200-110–receptor complexes were solubilized in digitonin from either rat brain nerve terminals or skeletal muscle microsomes, and incubated with sera, and immune complexes were detected by methods similar to those for the N-type channel immunoprecipitation assay described already. No immunoreactivity against either neuronal or skeletal muscle L-type calcium channels was detected in ALS sera (Table 1). We previously reported that this technique detects autoantibodies against neuronal and

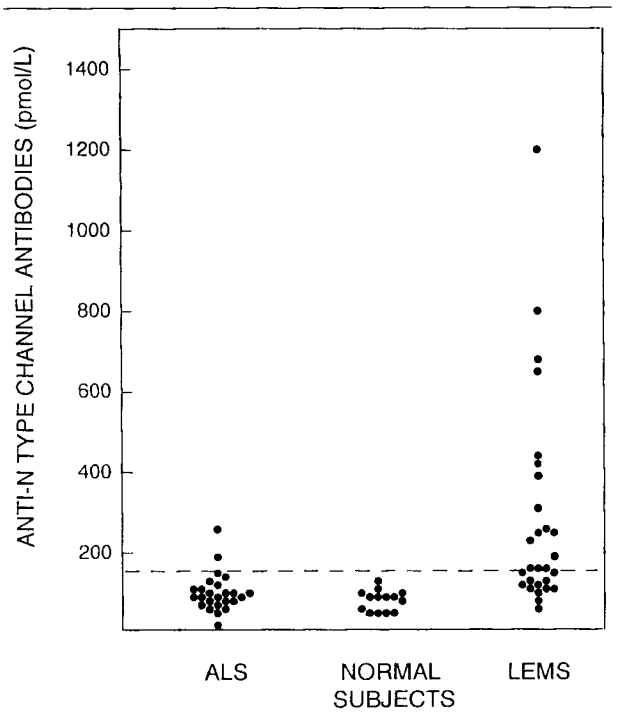

Fig 1. Immunoprecipitation assay for anti–N-type calcium channel autoantibodies. N-type calcium channels in rat brain membranes were labeled with ^{125}I-ωGVIA and extracted with Triton X-100. Extracts containing 6 to 8 fmol of labeled N-type calcium channel were incubated with 5 μl of serum from patients with amyotrophic lateral sclerosis (ALS) *(25) or Lambert-Eaton myasthenic syndrome* (LEMS) *(31) or from healthy subjects (14) for 4 hours at 4°C. The amount of radioactivity recovered in immune complexes was counted and antibody titer was expressed in picomoles of calcium channel immunoprecipitated per liter of serum.*

muscle L-type channels from some LEMS sera [16]. However, only sera from a small group of patients displaying exceptionally high titers of antibodies against N-type channels reacted with L-type channels. Two of these samples were included in the series of assays shown in Table 1 as positive controls.

Our immunoprecipitation procedure is potentially limited by the fact that certain human immunoglobulin classes do not bind to protein A, which could account for our failure to demonstrate significant levels of reactivity against either N-type or L-type calcium channels in sera from ALS patients. Assays for antibodies against both N- and L-type channels were therefore repeated using polyvalent anti–human IgG, A, and M antibodies bound to protein A–Sepharose beads to capture immune complexes. This method also failed to reveal anti–calcium channel antibodies with ALS sera, but with LEMS sera gave similar results to those obtained when secondary antibodies were omitted (not shown).

It has been proposed that ELISA may be a more sensitive technique than immunoprecipitation for the detection of autoantibodies in ALS sera [2]. Consequently ELISA was carried out using N-type channels, purified from rat brain by a method that we recently reported [19], as an antigen. Purified proteins were dialyzed to reduce detergent concentration and then adsorbed to the wells of microtitration plates. Following blocking and incubation with tenfold diluted sera, immune complexes were detected using an anti–human immunoglobulin–alkaline phosphatase conjugate. Sera from 2 ALS patients (8%) gave responses slightly above (0.28 and 0.26) the limit of the normal range (0.24), whereas sera from 12 LEMS patients (39%) were immunoreactive (Fig 2). Therefore, ELISA did not reveal a higher percentage of positive ALS sera than did the immunoprecipitation assay. Furthermore, a smaller number of LEMS sera were positive, suggesting that ELISA is a less sensitive technique than immunoprecip-

Table 1. Immunoprecipitation Assay for Anti–L-Type Calcium Channel Autoantibodies

	Amyotrophic Lateral Sclerosis	Normal Subjects	Lambert-Eaton Myasthenic Syndrome
Brain [^{3}H]PN200-110 receptors			
No. of patients	14	11	1[b]
No. of positive samples[a]	0	0	1
Mean titer (pmol/liter of serum ± SD)	84 ± 29	131 ± 40	684
Muscle [^{3}H]PN200-110 receptors			
No. of patients	24	18	1[b]
No. of positive samples[a]	0	0	1
Mean titer (pmol/liter of serum ± SD)	125 ± 30	151 ± 22	1,700

[a]Serum titers above the mean titer + 3 standard deviations (SDs) for the normal subjects were scored as positive.
[b]Serum from 2 Lambert-Eaton myasthenic syndrome patients previously reported to react with L-type calcium channels [15] were included as positive controls.

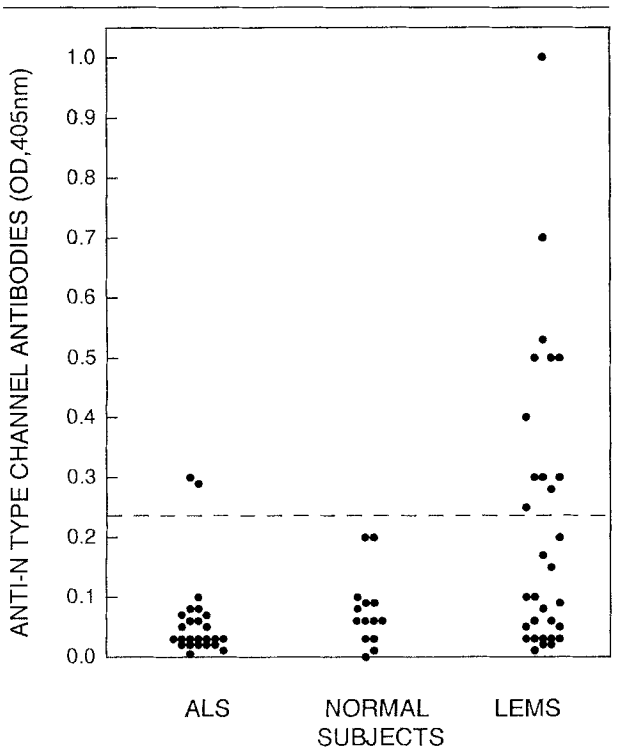

Fig 2. Enzyme-linked immunosorbent assay for anti–N-type calcium channel antibodies. N-type calcium channels were purified from rat brain membranes and adsorbed to microtitration plates (0.3 μg/well). After incubation for 2 hours at 37°C with tenfold-diluted serum from patients with amyotrophic lateral sclerosis (ALS) *(25) or Lambert-Eaton myasthenic syndrome* (LEMS) *(31) or from healthy subjects (14), and washing, immune complexes were detected with anti–human immunoglobulin antibodies conjugated to alkaline phosphatase. The optical density at 405 nm was measured 1 hour after the addition of 0.5 mg/ml of* p*-nitrophenyl phosphate.*

itation for the detection of autoantibodies against neuronal calcium channels.

Discussion

Reports from Appel and colleagues provided persuasive evidence that autoantibody-mediated activation of voltage-gated calcium channels may underly the degeneration of motor neurons in ALS. Immunoglobulins from ALS patients were shown to enhance acetylcholine release at the neuromuscular junction [3] and increase voltage-dependent calcium currents in cerebellar neurons [7], oocytes injected with rat brain messenger RNA [8], and a motor neuron cell line [9]. Surprisingly effects were not restricted to neurons, as ALS IgG inhibited calcium currents in skeletal muscle by shifting the activation curve to more positive potentials and stabilizing the closed state [21]. However, ELISA findings were consistent with functional effects and ELISA detected antibodies that bind to purified L-type calcium channels from skeletal muscle in 75% of patients with sporadic ALS [11].

More recent data from the Appel group show that ALS IgG mediated cytotoxicity in a hybrid motor neuron line which was prevented by either ω conotoxin GVIA or ω agatoxin IVA, selective antagonists of neuronal N- and P-type calcium channels, respectively [10]. Although no protection was afforded by L-type channel antagonists, neurotoxicity was reduced when ALS IgG was preincubated with purified L-type calcium channels from muscle, presumably due to the removal of a specific antibody population [10].

These observations suggest that cytotoxicity is initiated by ALS antibodies interacting directly with neuronal N- and P-type calcium channels, and also imply that these antibodies can cross-react with multiple types of calcium channel. However, direct evidence for binding of ALS IgG to neuronal calcium channels is sparse, although appropriate methods are available and anti–calcium channel autoantibodies in LEMS have been well documented by immunoassay in multiple independent studies [12–18, 22, 23]. We therefore performed assays using both immunoprecipitation and ELISA to compare anti–calcium channel reactivity of sera from patients with ALS or LEMS and from normal subjects.

The results obtained in assays for anti–N-type calcium channels are summarized in Table 2. Only 8% (2/25) of ALS patients had titers marginally above the upper limit of those in the normal subjects, whereas the assay method did reveal antibodies in the sera of 58% (18/31) of LEMS patients. It has been suggested that ELISA may be a more sensitive method than im-

Table 2. Detection of Anti–N-Type Calcium Channel Autoantibodies[a]

	Amyotrophic Lateral Sclerosis	Normal Subjects	Lambert-Eaton Myasthenic Syndrome
Group	25 (100)	14 (100)	31 (100)
Positive on immunoprecipitation assay	2 (8)	0	18 (58)
Positive on enzyme-linked immunosorbent assay	2 (8)	0	12 (39)
Positive on both assays	0	0	10 (32)
Negative on both assays	21 (84)	14 (100)	11 (35)

[a]Data are numbers of serum samples, with percentages in parentheses.

munoprecipitation for the detection of ALS antibodies that react with calcium channels [2]. It is indeed conceivable that low-affinity antibodies may be trapped by localized concentrations of a solid-phase antigen although they may fail to capture a dilute antigen in solution. Biochemical work in the 1980s relied on preparations from skeletal muscle microsomes, which contain a relatively high density of L-type channels [24–26]. However, calcium channels in nerve terminals are present at much lower densities and purification has only recently been achieved [19, 27, 28]. We used ωGVIA-sensitive N-type calcium channels, purified by a method that we recently reported [19], as an antigen in ELISA to attempt to detect autoantibodies in the same series of ALS and LEMS patients. The results summarized in Table 2 indicate that sera from 2 of 25 ALS patients and 12 of 31 LEMS patients contained reactive antibodies. The titers in the two positive ALS sera were both equivocally close to the upper limit of the titers in samples from healthy control subjects. Therefore, these data are not consistent with the view that ELISA is a more appropriate method to detect autoantibodies in ALS. Furthermore, ELISA was less sensitive in revealing autoantibodies in LEMS, as only 39% of the sera were positive compared to 58% in immunoprecipitation assays. Interestingly, although 10 (32%) of 31 LEMS sera reacted with N-type channels both in solution and in solid phase, none of the ALS sera were positive in both assays.

We recently reported that immunoprecipitation of solubilized [^{3}H]PN200-110–labeled receptors can be used to assay autoantibodies that bind to L-type calcium channels [16]. LEMS sera that are positive in this assay react with neuronal, cardiac, and skeletal muscle L-type calcium channels and with neuronal N-type channels [16], a lack of specificity similar to that observed by Appel's group in ALS sera. Consequently, ALS sera were screened for immunoprecipitation of neuronal or skeletal muscle L-type calcium channels, but no reactivity was detected in either assay.

We did not perform systematic immunoprecipitation assays using ^{125}I-ω conotoxin MVIIC (^{125}I-ωMVIIC), which binds to P/Q-type calcium channels and provides a sensitive test for autoantibodies in LEMS sera [17, 23]. However, preliminary experiments with a restricted group of ALS patients did not reveal reactive sera (not shown). A recent report showed that 22% of ALS sera were positive in this assay [23], but this was not confirmed in another study in which only 1 of 27 ALS sera gave a weakly positive response [29] (B. Lang, personal communication, 1996).

In conclusion, our results are not consistent with significant levels of autoantibodies directed against calcium channels in the sera of patients with sporadic ALS. Diagnosis was established according to the El Escorial World Federation of Neurology criteria [20], and it is therefore unlikely that the discrepancy between our findings and those of Appel and colleagues is due to our patients constituting a distinct group. Appel and colleagues reported antibodies against muscle L-type calcium channels in 75% of patients and hypothesized that these antibodies directly trigger cell toxicity by binding to neuronal N-type calcium channels [8, 10, 11]. We cannot rule out the possibility that ALS sera contain low-affinity autoantibodies against muscle L-type calcium channels that may only be assayed by ELISA. However, we did not detect immunoreactivity with N-type calcium channels using both immunoprecipitation assay and ELISA, indicating that if ALS sera do contain anti–muscle calcium channel autoantibodies, these antibodies do not cross-react with neuronal channels. Consequently, our data do not support the hypothesis that antibodies that bind to and activate neuronal calcium channels initiate events resulting in motor neuron degeneration in ALS.

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