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IgG from amyotrophic lateral sclerosis patients increases current through P-type calcium channels in mammalian cerebellar Purkinje cells and in isolated channel protein in lipid bilayer

(barium current/neurodegeneration/cell death)

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ABSTRACT **The effect of the IgG from amyotrophic lateral sclerosis (ALS) patients was tested on the voltage-dependent barium currents (I_{Ba}) in mammalian dissociated Purkinje cells and in isolated P-type calcium channels in lipid bilayers. Whole cell clamp of Purkinje cells demonstrates that ALS IgG increases the amplitude of I_{Ba} without modifying their voltage kinetics. This increased I_{Ba} could be blocked by a purified nonpeptide toxin from *Agelenopsis aperta* venom (purified funnel-web spider toxin) or by a synthetic polyamine analog (synthetic funnel-web spider toxin) and by a peptide toxin from the same spider venom, ω-Aga-IVA. Similar results were obtained on single-channel recordings from purified P channel protein. The addition of ALS IgG increased single-channel I_{Ba} open time without affecting slope conductance. The results described above were not seen with normal human IgG nor with boiled ALS IgG. It is concluded that ALS IgG enhances inward current through P-type calcium channels. Since P-type Ca^{2+} channels are present in motoneuron axon terminals, we propose that the enhanced calcium current triggered by ALS IgG may contribute to neuronal damage in ALS.**

Amyotrophic lateral sclerosis (ALS; "Lou Gehrig's disease") has been proposed to be an autoimmune disease that can attack upper and lower motoneurons (1), leading to paralysis, respiratory depression, and death. It has been previously reported that IgG from ALS patients has a blocking effect on L-type calcium channels (2) and in lipid bilayer (3). In addition, ALS IgG enhances calcium currents (I_{Ca}) in immortalized motoneurons (4), acting on an as yet unidentified calcium channel. This IgG has also been shown to produce an increase in acetylcholine release (5), a mechanism in which the P-type calcium channel is directly involved (6). Here, we demonstrate, using patch-clamp recordings from Purkinje cells (7, 8), that ALS IgG produces an increase in barium current (I_{Ba}). A parallel experiment using purified P-type calcium channels isolated from the cerebellum and incorporated into a lipid bilayer (9, 10) demonstrated that ALS IgG increased the open time of such P-type calcium channels. These results indicate the presence, in ALS patients, of an antibody that affects P-type calcium channels by increasing their open time, without affecting single-channel conductance.

METHODS

Purkinje Cells. Purkinje cells were isolated from adult guinea pig cerebellar cortex and dissociated using the Kay and Wong method (11). Following dissociation, cells were placed in a recording chamber and visualized with standard light microscopy. Inward currents were obtained under patch-clamp conditions in the presence of 1 μM tetrodotoxin and of 10 mM Ba replacing the usual 2.0 mM calcium in the bathing solution (12, 13). The patch electrode contained 110 mM tetraethylammonium chloride, 4 mM MgCl, 2 mM Tris ATP, 1 mM GTP, 14 mM creatinine phosphate, 9 mM Hepes, and 9 mM EGTA. Results were obtained using a Wermer patch-clamp amplifier (PC-501 patch clamp with a I-GΩ headstage; Hamden, CT). The barium current–voltage (*I–V*) relation was determined prior to the bath administration of ALS IgG or calcium blockers. Following initial measurements of I_{Ba} in dissociated Purkinje cells (n = 10), IgG was introduced directly to the bath at a concentration of 27 μg/ml (patient 1) or 16 μg/ml (patient 2). In addition to ALS IgGs, IgGs from normal subjects were utilized as controls. To determine whether the effects were due to ALS IgG, the IgG was boiled 20 min. In a second set of experiments IgG was warmed to 53°C for a period of 30 min to destroy the complement, while keeping intact the IgG fraction. P-channel blocker, purified funnel-web spider toxin (pFTX; 1 μl/ml) (9), synthetic FTX (sFTX; 1 mM) (10), ω-Aga-IVA peptide (50–100 nM) (14), ω-conotoxin (10 μM), and nifedipine (10 μM) (15) were utilized in the pharmacological studies.

Animals/Membranes for P-Channel Isolation. Adult Hartley guinea pigs (200–300 g) from Camm Research Institute (Wayne, NJ) were decapitated with a small animal guillotine under sodium pentobarbital (Nembutal, 40 mg/kg, i.p.) anesthesia. The brain was carefully excised and the cerebellum was dissected free of remaining brain tissue using a small spatula. The excised cerebellum from 6–12 animals was pooled and homogenized into 10 volumes of 400 mM sucrose/5 mM Tris·HCl/0.1% bacitracin/0.1% phenylmethylsulfonyl fluoride/5 mM EDTA/≈2 units of aprotinin at 4°C until a uniform homogenate was obtained. The homogenate was then subjected to differential centrifugation at 700 × *g* for 10 min. The supernatant was recovered and subjected to further centrifugation at 12,000 × *g* for 15 min.

The supernatant was again subjected to centrifugation at 47,000 × *g* for 30 min. The pellet was saved and was resuspended into 100 mM sodium citrate buffer (pH 7.4) to a protein concentration of ≈20 mg/ml as determined by Lowry assay. This solution was then brought to 3% sodium choleate based on the total solution volume. The solution was stirred overnight at 4°C to solubilize protein and was then centri-

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Abbreviations: ALS, amyotrophic lateral sclerosis; *I*, current; *V*, voltage; FTX, funnel-web spider toxin; pFTX, purified FTX; sFTX, synthetic FTX.

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fuged at 47,000 × g for 30 min. The supernatant was subjected to affinity purification as described below.

Affinity Gel Preparation and Protein Purification. An affinity gel based on sFTX was synthesized as described (9, 10). The solubilized protein solution was applied to the polyamine-coupled gel and was stirred overnight at 4°C. The gel mixture was washed with 10 volumes of sodium citrate (100 mM, pH 7.4) and was vacuum filtered. To remove the bound protein the gel cake was resuspended into 100 ml of 1 M $CaCl_2$ (pH 7.0) containing 1% sodium choleate and was stirred in a cold room for 2 hr. The preparation was vacuum filtered and the filtrate was collected. The gel was again suspended in the $CaCl_2$/choleate solution and the elution procedure was repeated. The eluates were pooled and dialyzed for 24 hr against 100 mM sodium citrate (pH 7.4) to reduce the calcium and detergent concentrations. This solution was then concentrated to a small volume by dialysis against polyethylene glycol (M_r 35,000). A portion of this solution was taken for SDS/polyacrylamide gel electrophoresis and the remainder was reconstituted into lipid vesicles.

Vesicle Formation. Lipid vesicles containing the isolated P-channel protein were formed using a 4:1 mixture of phosphatidylethanolamine/phosphatidylcholine in 400 mM sucrose containing 1% sodium choleate by sonication and dialysis as described by Racker (16).

Lipid Bilayer Studies. The functional activity of the P-channel protein purified as described above was studied using the "tip-dip" bilayer technique (17). Bilayers were formed on two-pull micropipettes with opening diameters of ≈1 μm using a 1:1 mixture of phosphatidylserine/phosphatidylethanolamine. Voltage was applied via the micropipette using an Axon Instruments AI 2120 patch clamp with a 100-GΩ headstage. The bathing solution was held at ground.

Data obtained in the channel studies using P-channel protein preparations of cerebellar origin were amplified to 1 V/pA and the membrane current was recorded. To obtain amplification of the Ca^{2+} channel response, a solution containing 80 mM $BaCl_2$ (pH 7.2) was used on the pipette side of the membrane and a solution containing 120 mM CsCl (pH 7.2) was used on the bath side. The applied voltage (holding potential) ranged from −200 mV to +200 mV. In experiments testing the effects of IgGs obtained from patients with ALS, dilutions of 1:1000 and 1:10,000 were prepared in 80 mM $BaCl_2$ and were used to fill a fresh micropipette. Additionally, experiments were performed using IgGs that had been complement-inactivated or totally inactivated by using the heating protocols previously described. Prior to each test of the IgGs, control recordings using pipettes containing only $BaCl_2$ (pH 7.2) were obtained. Data was filtered at 1000 Hz and analyzed using the PCLAMP suite of analysis programs (Axon Instruments).

Isolation of IgG from ALS Patients. IgG was purified by previously described methods (18) and stored at −80°C until use. IgG content in purified fractions was typically >90%, as measured densitometrically from Coomassie-stained SDS/PAGE electrophoretic profiles of IgG fractions and by IgG immunoprecipitation using a Technicon RA-1000 system analyzer (Technicon).

RESULTS

Patch-Clamping of Dissociated Purkinje Cells. Barium currents obtained from a dissociated Purkinje cell with extracellular Ba concentrations of 10 mM are illustrated in Fig. 1 *A–D*. These currents were obtained following various amplitude voltage steps from a holding of −70 mV. The currents demonstrate the typical slow onset, lack of inactivation, and fast tail current observed in Purkinje cells (7, 8). In Fig. 1*A* the central inward current (control) is determined prior to the addition of ALS IgG. Fifteen minutes after application of the ALS IgG, the same voltage step demonstrates an increased barium current. Note the significant increase in the amplitude of the sustained calcium current as well as in amplitude of the tail current. A detailed *I–V* family for I_{Ba} before (Fig. 1*B*) and

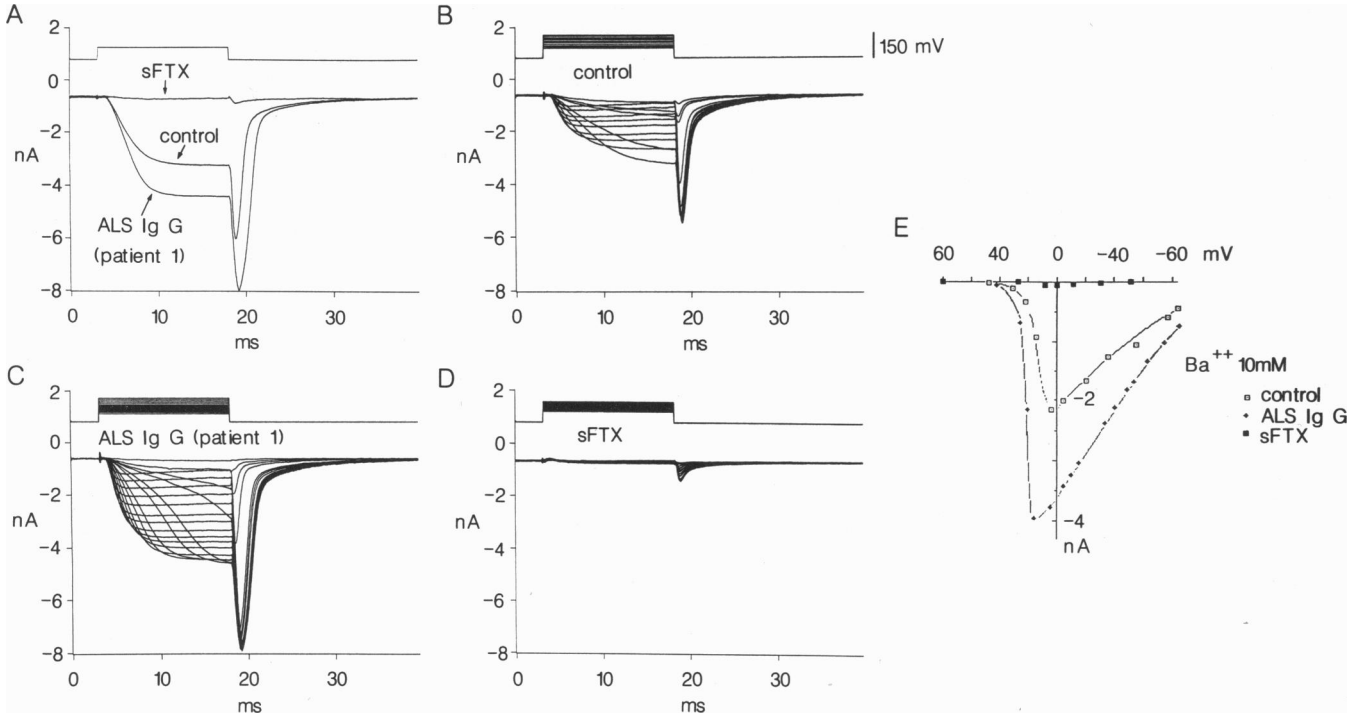

FIG. 1. Whole cell patch-clamp recording of I_{Ba} from guinea pig Purkinje cells in the slice. (*A*) Lower set of traces, control I_{Ba} produced by voltage step of 60 mV from a holding of −70 mV. Following the application of ALS IgG, I_{Ba} increased in amplitude. Application of sFTX to the bath abolishes I_{Ba}. (*B–D*) Family of I_{Ba} for different voltage steps for control (*B*), ALS IgG superfusion (*C*), and after sFTX superfusion (*D*). (*E*) *I–V* relations for the set of records shown in *B–D*.

after ALS IgG (Fig. 1*C*) demonstrates that the increase in barium current is present particularly at levels positive to −40 mV membrane potential. Application of sFTX (Fig. 1 *A* and *D*) demonstrates that this polyamine blocks the inward Ba current. In Fig. 1*E*, comparison of the *I–V* relation for the control I_{Ba} and that obtained following IgG perfusions is shown. Open circles represent the *I–V* relation for the control levels and closed circles represent the relation following IgG administration. These records are typical for the findings obtained in 10 neurons and indicate that the calcium current is increased in the presence of IgG.

A similar set of recordings obtained for a second patient in two other Purkinje cells is shown in Fig. 2*A*. As in the results illustrated in Fig. 1, the I_{Ba} was blocked by sFTX. In addition, 50 and 100 μM ω-Aga-IVA produced an equally dramatic block of the Ba where the difference between control of barium level and that obtained 12 min after application of ALS IgG is illustrated for another cell.

P-Type Channels Are Involved in the Increased Calcium Entry. To determine the type of voltage-dependent calcium channel responsible for the increase in I_{Ba}, pFTX, sFTX (known to block P channels) (9, 19), and ω-Aga-IVA peptide (14) were tested for I_{Ba}-blocking ability. Following applications of ALS IgG, sFTX (at 500 μM) produced a total block of the barium current (Figs. 1 *A*, *D*, and *F* and 2 *A* and *B*). In the record illustrated in Fig. 2*B*, a set of results similar to those in Fig. 2 *A* and *C* was obtained following ω-Aga-IVA superfusion. Barium current in this case was blocked by ω-Aga toxin IVA at a concentration of 100 nM.

To verify that the above results were due to the presence of ALS IgGs, a similar set of results was obtained using boiled IgGs (Fig. 3*A*), which produced no demonstrable effect on barium currents. By contrast, ALS IgG heated to 53°C, to inactivate complement, demonstrated a clear increase of calcium current, which once again was blocked by pFTX at a concentration of 1 μl/ml. Bath application of normal IgG in similar concentration did not produce any change in I_{Ba} (Fig. 3*C*). To substantiate further our conclusion regarding P-channel activation as the possible underlying mechanism for the increased ALS current, ω-conotoxin and nifedipine were also used at 10 μM and 10 μM, respectively. These blockers had no effect on I_{Ba}, confirming the P-type origin of the calcium conductances in Purkinje cells (9, 13).

Lipid Bilayer Results. As previously demonstrated (9, 10), protein isolated from the cerebellar cortex using a sFTX-based affinity gel exhibited single-channel events when vesicles incorporating the purified protein were studied using the lipid bilayer technique. Fig. 4*A* shows the recordings obtained when vesicles incorporating purified P-channel protein were fused with a lipid bilayer in the absence and presence of IgG obtained from an ALS patient.

In Fig. 4*A*, normal recordings were obtained at holding potentials of −60, −50, −30, and −10 mV in asymmetric 80 mM $BaCl_2$/120 mM CsCl solutions. At −60 mV, few if any openings were observed. Beginning at −50 mV, channel-like events could be detected, which increased in frequency in a voltage-dependent manner as the membrane was further depolarized. Thus, open probability increases with depolarization. However, the open times of the individual events remain short, with a mean open time of 1 ms (9, 13), and are consistent with the properties of the P-type calcium channel responsible for the calcium conductance of mammalian Purkinje cells.

Fig. 4*B* shows the dramatically different recordings that were obtained when ALS IgG at a concentration of 3 μg/ml was present at the cytoplasmic face of the P-type calcium channel. The recording obtained at a holding potential of −60 mV shows that the threshold for activation of the channel had not been altered and that little or no activity was apparent. As the membrane was depolarized, channel-like events were observed. Recordings at −50 mV remain similar to those obtained for this channel in the absence of the IgG. With further depolarization, events were seen that are remarkable for the duration of their open time, which was increased manyfold without any alteration of the single-channel conductance. Determination of the single-channel current indi-

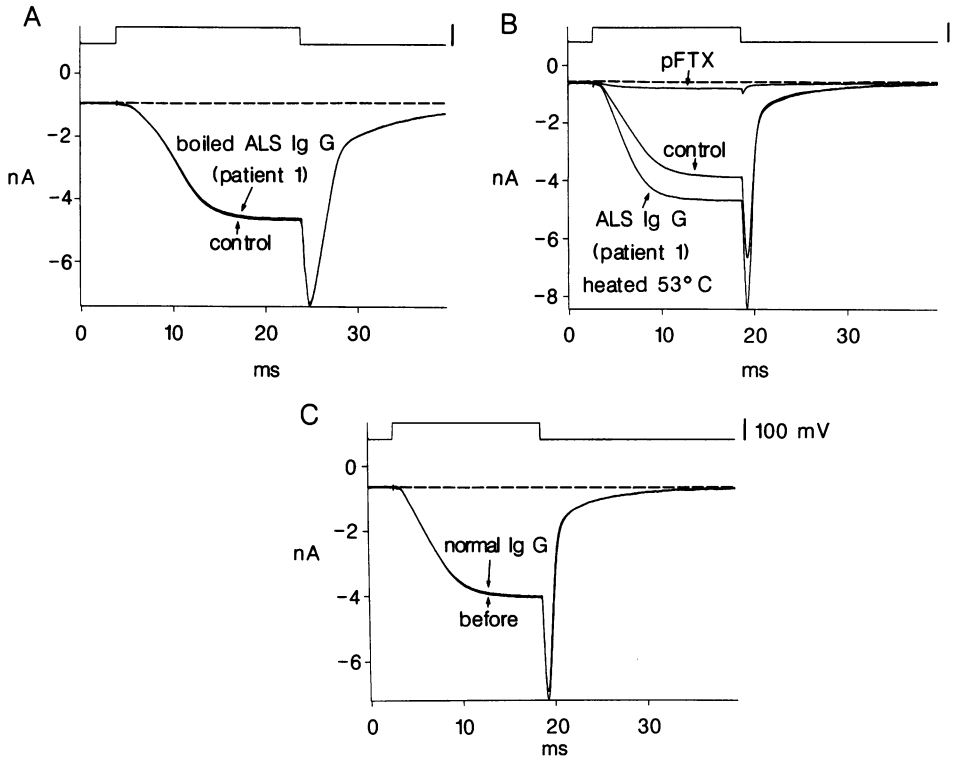

FIG. 2. Purkinje cell I_{Ba}. (*A*) Effect of boiled ALS IgG compared to the control condition. (*B*) Effect of IgG heated 53°C and the action of pFTX on I_{Ba}. (*C*) Effect of normal IgG on I_{Ba}. Holding potentials were the same as in Fig. 1.

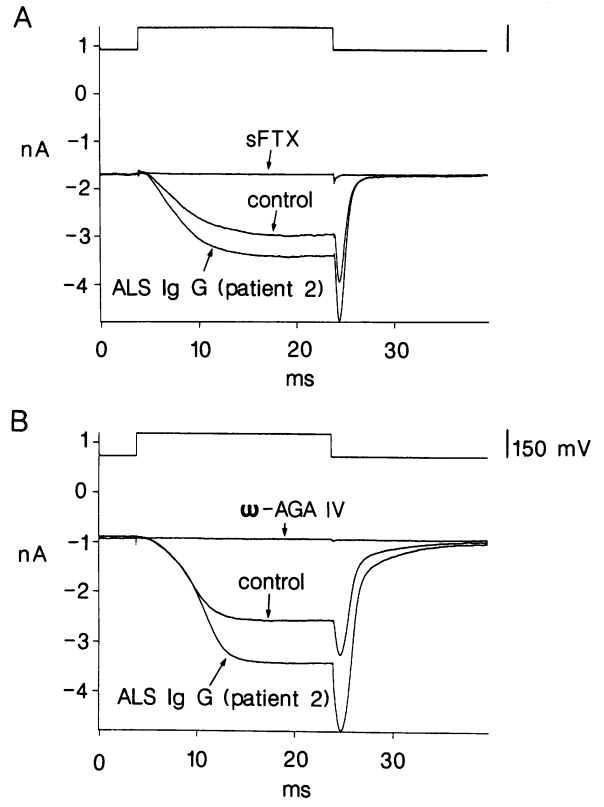

FIG. 3. Purkinje cell I_{Ba}. (*A*) Control I_{Ba} is increased after exposure to ALS IgG from a second patient. I_{Ba} is blocked by sFTX. (*B*) Similar set of recordings as in *A* from a different neuron using the same IgG but blocked by ω-Aga-IVA.

cates that ALS IgG produced no increase in channel conductance (Fig. 4*C*). IgGs from normal subjects and boiled ALS IgG were without effect in these experiments. These results are taken to indicate that ALS IgG acts directly on channel protein, rather than by other mechanisms involving intracellular machinery known to regulate single-channel-calcium properties.

DISCUSSION

The present findings indicate that IgG from ALS patients acts directly on P-type channels and modifies the average open time such that a rather large increase in I_{Ba} may be observed. This increase does not seem to modify kinetics of the onset of calcium currents and has little effect on the duration of the tail current. This last point, however, cannot be qualitatively interpreted as dissociated neurons retain dendritic branches that prevent a proper control of I_{Ba} currents, especially at the break of the voltage pulse. The results indicate, nevertheless, that ALS IgG as well as complement-inactivated serum are capable of producing the I_{Ba} increase, whereas normal or boiled serum does not modify I_{Ba}.

From the above it may be concluded that IgG affects the well-known P-type calcium currents in Purkinje cells. To confirm this finding, pFTX from *Agelenopsis Aperta*, sFTX, and ω-Aga-IVA toxin were tested and showed calcium-current block in the absence and presence of ALS IgG. These results confirm that pFTX, sFTX (9, 13), and ω-Aga-IVA (14) can block barium currents in Purkinje cells and demonstrate that the increase of calcium current is brought about by activation of P-channel types of calcium conductance. The fact that the increased I_{Ba} was insensitive to nifedipine as well as to ω-conotoxin rules out the possibility that these currents could be due to activation of an N- or L-type calcium channel (15) and may help to explain the increase in I_{Ca} obtained with ALS IgG in other preparations such as immortalized motoneurons (4).

Finally, results on the lipid bilayer indicate that the effect of ALS IgG on P channels is direct, since in the lipid bilayer

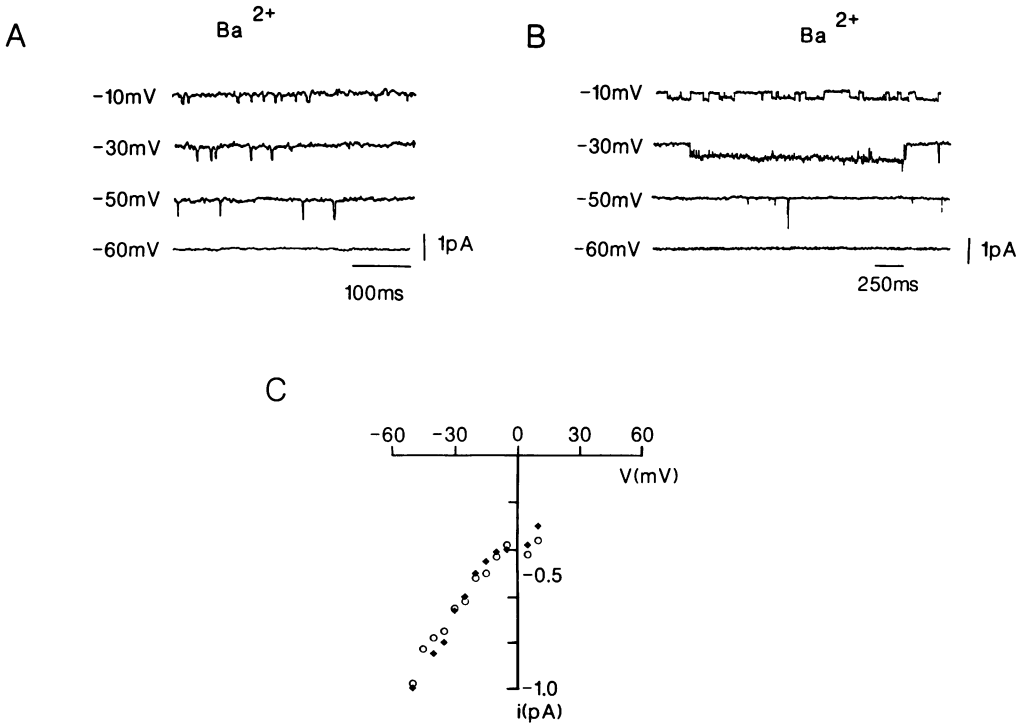

FIG. 4. Single-channel recording from isolated P-channel protein in the lipid bilayer. (*A*) Voltage-dependent single-channel recordings. (*B*) ALS IgG increases open time of single-channel current. Note the change in time base for *A* and *B*. (*C*) *I–V* relations for normal and ALS IgG-treated single-channel currents.

preparation only the channels responsible for calcium current are actually present. Of interest at this level is the fact that in the presence of ALS IgG the open time for single calcium channels appears to be voltage-sensitive such that as the membrane is depolarized, the average open time substantially increases. This is to be expected as multiple openings often coexisted (not illustrated). This may help to explain to a certain extent why this time constant of the tail current increased by 100% only in the presence of IgG in dissociated Purkinje cells, even though very large conductance open events do not seem to be evident as might be expected. It must also be remembered, however, that in the real cell, as opposed to the isolated channel in lipid bilayers, other changes may enter into consideration.

The present findings are generally in agreement with those previously reported for the actions of ALS IgG on ionic channels. In those studies, it was demonstrated that the IgG fraction blocks skeletal L-type calcium channels (2, 3). However, as it is known that ALS IgG produces an increase in acetylcholine release (5) and that in the mammal this release is under the control of P-type channels (6), block of L-type calcium channels could not explain the pathology seen in ALS.

The ability of IgG from ALS patients to act on multiple types of calcium channels is of interest. In addition to the present findings regarding the activation of P-type calcium channels and previous findings on the block of L-type calcium channels, the IgG fraction has also been shown to alter the behavior of ω-conotoxin-sensitive channels in oocytes (E.S. and S.A., unpublished observation). These findings, when taken together, suggest that either ALS patients produce multiple antibodies that act on different calcium channel proteins or, more attractively, that a single antibody is being produced that acts on an identical epitope present on multiple channel types. In the absence of a resolved antibody preparation this question cannot be fully addressed. It is also not clear from these results whether the antibodies produced by these patients are uniquely directed toward calcium channels or if the action is secondary to a different immunologic target.

The results, however, are quite unambiguous in demonstrating that ALS IgG acts directly on P-type channels, allowing increased calcium to enter cells, and may go a long way toward explaining cell degeneration in this syndrome. In particular, the possibility remains that autoimmune events may trigger a similar type of sustained calcium inflow in other neurons since the P channel is present throughout the central nervous system (20). Such calcium-channel misregulations may be at the base of many degenerative diseases of the central nervous system, as excessive intracellular calcium concentrations are well-known to result in cell death, as is the case in Purkinje cells (21), although the presence of high levels of calcium buffer protein may actually protect such cells from early damage.

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