Anti-Ca\textsuperscript{2+} channel antibody attenuates Ca\textsuperscript{2+} currents and mimics cerebellar ataxia \textit{in vivo}

Yaping Joyce Liao\textsuperscript{*†‡}, Parsa Safa*, Yi-Ren Chen*, Raymond A. Sobel§, Edward S. Boyden*, and Richard W. Tsien\textsuperscript*‡

Departments of *Molecular and Cellular Physiology, \textsuperscript{†}Ophthalmology and Neurology, and \textsuperscript{§}Pathology, Stanford University School of Medicine, Stanford, CA 94305

Contributed by Richard W. Tsien, November 16, 2007 (sent for review August 20, 2007)

Voltage-gated Ca\textsuperscript{2+} channels (VGCCs) are membrane proteins that determine the activity and survival of neurons, and mutations in the P/Q-type VGCCs are known to cause cerebellar ataxia. VGCC dysfunction may also underlie acquired peripheral and central nervous system diseases associated with small-cell lung cancer, including Lambert–Eaton myasthenic syndrome (LEMS) and paraneoplastic cerebellar ataxia (PCA). The pathogenic role of anti-VGCC antibody in LEMS is well established. Although anti-VGCC antibody is also found in a significant fraction of PCA patients, its contribution to PCA is unclear. Using a polyclonal peptide antibody against a major epitope in the P/Q-type VGCC and evaluated its serum followed by toxin, led to an occlusion of the GVIA effect (Fig. 1D–F), consistent with a competition between the D-III antibody and GVIA for N-type VGCCs. These results would make sense if antibody and GVIA both bound near the permeation pathway. However, the presence of D-III antibody did not abolish inhibition by \textomega-Aga-IVA (Fig. 1E and F), known to affect channel gating by binding to a site other than the pore (21).

Antibody Inhibition of Heterologously Expressed Ca\textsuperscript{2+} Channels. Having obtained these tantalizing results in cerebellar granule neurons, we set out to study the mechanism of antibody action more specifically. We purified the IgG fraction of the rabbit serum and assessed its effects on human embryonic kidney (HEK293) cells expressing only one type of VGCC (Fig. 24). The D-III IgG inhibited N-type Ca\textsuperscript{2+} current most significantly, reaching 41.0 ± 0.9% inhibition at 3 mg/ml (IC\textsubscript{50} = 115 µg/ml) (Fig. 2B), whereas control IgG had no effect (data not shown). D-III IgG also inhibited P/Q-type VGCCs, although less effectively (20.1 ± 2.6% inhibition at 3 mg/ml, IC\textsubscript{50} = 245 µg/ml). D-III IgG had no effect on L-type Ca\textsuperscript{2+} current at 3 mg/ml.

Because the D-III epitope is located near the GVIA-binding site, we looked further for possible competition between the D-III antibody and GVIA. Indeed, in HEK293 cells expressing N-type VGCCs, GVIA was significantly less effective in the presence of D-III IgG (Fig. 2C); the time constant (\(\tau\)) of current reduction was 534 ± 245 s, a 10-fold increase over that found with GVIA alone (\(\tau = 51.5 ± 3.8\) s). There was no competition between D-III IgG (1 or 3 mg/ml) and \textomega-Aga-IVA (500 nM) in HEK293 cells expressing P/Q-type VGCCs (data not shown). The competition between the D-III IgG and GVIA but not \textomega-Aga-IVA provided further evidence that the D-III antibody

Results

Functional Effects of Anti-VGCC Antibody. We looked for a functional effect of the Domain-III S5–S6 loop (D-III) antibody by using whole-cell voltage-clamp recordings of cerebellar granule cells, known to express N-, P/Q-, L-, and R-type VGCCs (20). The D-III serum (1:100 dilution) inhibited the Ca\textsuperscript{2+} current rapidly and reversibly (Fig. 1B). The sensitivity of the Ca\textsuperscript{2+} current to \textomega-conotoxin-GVIA (GVIA) and \textomega-agatoxin-IVA (\textomega-Aga-IVA) demonstrated the presence of N- and P/Q-type VGCCs, respectively, in the same neuron (Fig. 1B). After inhibition of both N- and P/Q-type VGCCs, the D-III serum had no further effect, indicating that it spared non-N- and P/Q-type Ca\textsuperscript{2+} currents. Preblock by \textomega-Aga-IVA did not abolish antibody inhibition of Ca\textsuperscript{2+} currents (Fig. 1D); thus, the D-III antibody did not inhibit solely P/Q-type VGCCs. Preblock with GVIA indicated that the D-III antibody did not selectively inhibit N-type VGCCs (data not shown). Taken together, these data suggest that the D-III antibody inhibited both N- and P/Q-type VGCCs.

Interestingly, the converse experiment, preblock by the D-III serum followed by toxin, led to an occlusion of the GVIA effect (Fig. 1D–F), consistent with a competition between the D-III antibody and GVIA for N-type VGCCs. These results would make sense if antibody and GVIA both bound near the permeation pathway. However, the presence of D-III antibody did not abolish inhibition by \textomega-Aga-IVA (Fig. 1E and F), known to affect channel gating by binding to a site other than the pore (21).

The authors declare no conflict of interest.


\*To whom correspondence may be addressed. E-mail: yjliao@stanford.edu or rwtsien@stanford.edu.

© 2008 by The National Academy of Sciences of the USA
exerted inhibitory effects on VGCCs by interacting with residues near the channel pore. Overall, the findings in HEK cells were entirely consistent with the data in the cerebellar granule neurons.

**Inhibition of VGCC in Presynaptic Terminals.** With the useful precedent of LEMS in mind, we wanted to know whether at central synapses, as at the neuromuscular junction, anti-VGCC antibody affects presynaptic function. At the synaptic output of the cerebellar granule axons, the parallel fiber–Purkinje cell synapse, presynaptic P/Q- and N-type VGCCs are the main regulators of neurotransmitter release (12). Excitatory postsynaptic currents (EPSCs), recorded in the Purkinje cell soma under whole-cell voltage-clamp, were rapidly inhibited by the D-III IgG current density by 26% and occluded further block by GVIA. These IgG experiments confirmed our early studies with D-III mice, which is 100% identical to P/Q-type and 85% identical to N-type VGCCs, typical of the high homology these channels share (41).

To ascertain the cause of the poor motor performance by the D-III mice, we digitally analyzed the mouse gait during the Rota Rod test. Compared with control mice, the D-III mice exhibited a distinctly irregular gait pattern (Fig. 4 D), often with visibly widened interhind paw distance. In this abnormal gait, the D-III mice took more steps to stay on the rod (Fig. 4 E) and were more likely to take short steps or skips, defined as step duration lasting \( \leq 0.5 \text{ s} \) (Fig. 4 F). The skips often occurred in series and sometimes in a direction that facilitated falling. This gait could not be attributed to decreased locomotive drive (Fig. 4 G) or to weakness (Fig. 4 H). However, the overall pattern of irregular gait and poor motor planning was strikingly similar to the abnormal motor behavior observed in PCA patients.

**Discussion**

Here, we have described the pathogenic actions of a functional peptide antibody, designed to target a disease-relevant, biophysically critical portion of the P/Q- and N-type VGCCs. We showed that antibody against the D-III region specifically inhibited somatic VGCCs in transfected HEK293 cells and cerebellar granule neurons and impaired synaptic transmission in the acute cerebellar slice by its action on presynaptic VGCCs. This inhibition of synaptic transmission could have occurred by a reduction in single channel current, channel number, or both. The
ability of the D-III antibody to slow the onset of action of GVIA by 10-fold is particularly notable because GVIA also binds to residues in the D-III region, the same region that encompasses the antibody epitope. Together, these findings strongly imply that the D-III antibody, like GVIA, binds near the channel pore. To look for effects of antibody in vivo, simulating what may happen in human disease, the D-III antibody was infused over the cerebellum, therefore bypassing the blood–brain barrier and avoiding the potential confounding behavioral effects of anti-VGCC antibody at the neuromuscular junction. Mice infused with D-III antibody exhibited cerebellar ataxia, characterized by irregular and excessive steps and skips. This altered motor behavior was observed in the absence of inflammation or neurodegeneration, consistent with a disease model where an antibody-mediated disruption of neurophysiology plays a major role in the pathogenesis of neurological symptoms.

We describe here a successful passive transfer model of a central nervous system channelopathy generated by anti-VGCC antibody, and we strongly support the significant role of antibody in the pathogenesis of PCA. Our work is complementary to human IgG passive-transfer models, both of LEMS (24, 25) and of cerebellar ataxia associated with antibody against metabotropic glutamate receptors (26). Our work establishes the central pathogenicity of antibody against the D-III region. The high homology between P/Q- and N-type VGCCs in this region supports one explanation for the ability of sera from LEMS patients to inhibit more than one type of high-voltage-activated Ca2+ channels: the antibodies target a well conserved epitope (27). In contrast, antibody against D-IV (22, 23), another epitope commonly found in LEMS patients (28), neither reduced Ca2+ influx into parallel fiber terminals in cerebellar slices nor caused loss of motor ability in vivo. Our results were consistent with the finding that the D-IV antibody failed to bind to intact cells derived from small-cell lung cancer, but only to solubilized cells (23). This pattern of domain-specific pathogenicity parallels findings in animal models of LEMS, where immunization with a D-III but not a D-IV peptide successfully replicates the peripheral disease (23, 29).

Our data highlight the importance of the D-III region, an epitope common to both N- and P/Q-type VGCCs. Historically, the first antibody associated with LEMS and PCA is against VGCC labeled by GVIA (N-type channel) (30, 31), although antibody against uniquely N-type epitopes is not likely to contribute to the pathogenesis of LEMS because of the predominance of P/Q-type VGCCs after development of the neuromuscular junction is completed (32). Because the N- and P/Q-type VGCCs are both present and important for synaptic transmission in the central nervous system (12, 33, 34), antibodies against
Fig. 4. Mice with brain infusion of D-III IgG exhibited cerebellar ataxia in the absence of neurodegeneration. (A) Mouse brain after infusion of a test dye, Fast green, over the cerebellum. Red arrow, site of cannula implantation; CTx, cerebral cortex; CB, cerebellum. (B) Montage of a coronal section of the postinfusion cerebellum stained with goat anti-rabbit IgG (green). BS, brainstem; 4th vent, fourth ventricle. (C–H) Blue, control IgG-infused mice; red, D-III IgG-infused mice. n = 7 unless otherwise stated. (C) Motor performance on the accelerated Rota Rod in nine trials performed over 3 days of IgG infusion. Longer fall latency corresponds to superior motor ability. The D-III mice performed 30% less well on the accelerated Rota Rod test than control mice (P = 0.04, repetitive ANOVA). (D) Representative 10-s digitized mouse gait during the Rota Rod test for a control mouse (Upper) and a D-III mouse (Lower). Each circle denotes the position of a hind paw, starting at 21 s into the Rota Rod test and ending with a fall for the D-III mouse 10 s later. The control mouse stayed on the rod for another 90 s (data not shown). Because the rod rotates downward, the hind paw position gradually decreased with respect to time as the mice stood on the rod. Open circles denote the paw position right after the mouse took a step. (E) Number of steps per min on the Rota Rod. D-III, 70 ± 4 steps per min vs. control, 53 ± 3 steps per min; *, P < 0.02. (F) Number of skips per min on the Rota Rod. D-III, 13.3 ± 2.9 skips per min vs. control, 1.7 ± 0.9 skips per min; **, P = 0.002. (G) Percentage time spent ambulating in open field. D-III, 34 ± 5% vs. control, 36 ± 6%, two trials per mouse. P = 0.75. (H) Frequency of vertical stance (free standing on fully extended hind limbs). D-III, 1.5 ± 0.4 times per min vs. control, 1.3 ± 0.3 times per min, two trials per mouse; P = 0.71. (C and E–H) Error bars are SEM. (I and J) Absence of TUNEL staining for dying neurons in the cerebellar cortex from control mice (n = 4) (I) and D-III mice (n = 3) (K). (L and L) Transmission electron microscopy shows normal morphology of Purkinje cells from the cerebellum of control (J) and D-III (L) mice. n = 3; mol, molecular layer; Pur, Purkinje cell layer; gr, granule cell layer; nuc, nucleus; cyto, cytoplasm. [Scale bars: 25 μm (I and K); 0.25 μm (J and L).]
epitopes that are uniquely specific to N-type or P/Q-type or shared by both VGCCs may contribute to PCA. This last possibility is highly likely given the significant degree of homology between the pore-forming subunits of N- and P/Q-type VGCCs. In the particular case of the D-III epitope, which is 85% identical between the two channel types, antibody block of N-type channels was at least as great as that of P/Q-type channels. Given the presence of antibodies against both kinds of VGCCs in the cerebrospinal fluid of PCA patients (16, 17), it seems likely that reductions in both N- and P/Q-type Ca2⁺ entry may contribute to the observed motor behavior.

Interestingly, the actions of the D-III antibody on N- and P/Q-type channels were reminiscent of the effects of the spider toxin ω-Aga-III, which caused only partial block of N-type channels even at maximal effective doses, but it rendered the remaining N-type current impervious to further blockade by GVIA (21, 35), as if it had occupied part of the GVIA-binding site. In a further parallel, both D-III antibody and ω-Aga-III caused only partial inhibition of P/Q-type channels at saturating concentrations but failed to prevent further reduction of current by ω-Aga-IVA, a gating modifier (21, 35). Nothing is published about the structural basis of ω-Aga-III binding, but one can be reasonably confident that D-III antibody interacts with the S5–S6 region of Domain-III, in close proximity to structural determinants of GVIA binding (37, 38). Furthermore, we found that binding of D-III antibody slows the on-rate for GVIA, as expected for direct competition, rather than an antagonistic allosteric action merely involving speeding of inhibitor off-rate. Taken together, the evidence lends strong support to a scenario whereby the partial inhibitor (in this case D-III antibody) occupies a structural determinant important for inhibitory transmission. Glass electrodes (1.2–2.0 MΩ) containing internal solution with 150 mM cesium methanesulfonate, 8 mM NaCl, 2.4 mM CaCl₂, 1.3 mM MgCl₂, pH 7.3, 310 mOsm. The slices were allowed to recover for at least 1 hour in oxygenated artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 2.4 mM CaCl₂, 1.3 mM MgCl₂, pH 7.3, 310 mOsm. Before recording in the presence of 10 μM gabazine (Tocris) to block inhibitory transmission, Glass electrodes (1.2–2.0 MΩ) containing internal solution with 150 mM cesium methanesulfonate, 8 mM NaCl, 0.1 mM CaCl₂, 0.6 mM MgCl₂, 1 mM EGTA, 4 mM Mg-ATP, 0.4 mM NaN₃, 0.1 mM D600, and 2 mM QX314, pH 7.25, 305 mOsm was used to record from Purkinje cells in whole-cell recording configuration. Low-intensity current was applied through a microelectrode (A.M.P.I. stimulator), while EPSCs were low-pass-filtered at 5 kHz and digitized at 10 kHz (Axopatch 10 amplifier, pClamp; Digidata). Leak and access resistance were monitored continuously, and experiments were rejected if these parameters changed significantly. The data were analyzed with custom-written programs in IgorPro (WaveMetrics) and Microsoft Excel.

Brain Infusion. All animal care and handling were performed in accordance with guidelines of the Stanford Institutional Animal Care and Use Committee. All steps of surgery were performed by an investigator blinded to the identity of IgG infused. Ten-week-old male C57BL/6 mice (weight > 20 g) were anesthetized with an i.p. injection of ketamine and meditomidine and placed in a stereotaxic apparatus (Kopf Instruments) with the body on top of a feedback-controlled heating pad to maintain core temperature at 37°C. Intermittent saline and oxygen were delivered nasally at 1–2 liters/min. Full recovery, behavior, and body temperature of the mice were repeatedly assessed over the 5- to 10-min operative period. Each mouse had s.c. injections of lacedt Ringer’s solution to prevent dehydration and a single injection of atropine to reduce mucous secretion. The Alzet 30-g infusion cannula (custom tube length, 1.5 mm) was implanted into the subarachnoid space above the rostral, dorsal cerebellum, ~3 mm caudal to lambda at midline. The Alzet osmotic pump (1003D, which delivers 1 μl/h over 3 days; Durect Corp.) was filled with control or experimental rabbit IgG (4–6 mg of IgG in 100 μl of PBS) and placed between the scalp. The amount of IgG infused was within the range of the Ca2⁺ channel IgG concentration found in the cerebral spinal fluid of patients with cerebellar ataxia associated with small-cell lung cancer (16). The cannula was secured with cyanoacrylate (VetBond; 3M) followed by dental cement (Hygenic), and the skin was closed with sutures. Intrapertoneal buprenorphine was given after surgery to reduce discomfort. Animals were monitored postoperatively until mobile, and at least twice daily. Multiple practice surgeries to infuse a low-molecular-weight dye Fast Green confirmed consistent delivery of the pump content (Fig. 4A). The delivery of rabbit IgG was confirmed after infusion with Alexa 488-labeled goat anti-rabbit IgG (Molecular Probes) staining of coronal sections through the cerebellum.

Behavioral Assessment. The behavioral experiments were carried out in an isolation room by an investigator blinded to the identity of infused IgG. Before surgery, the mice were habituated to the stationary Rota Rod once per day for 3 days, including the day of surgery, and once to the cage for open-field
locomotion. The mice were allowed to recover for ~15 h after surgery, and their behavior was assessed once per day for 3 days. Each of the behavioral tests was administered at the same time of the day throughout. The accelerated Rota Rod (Econometrics; Columbus Instruments) was used to assess locomotive ability and learning, and three trials were done per day for 3 days (total of 9 trials) with a baseline speed of 4 rpm and acceleration of 0.1 rpm/s and 2–3 min of rest between trials in the same day. After the behavioral experiments were completed, video recordings of the Rota Rod performance of mice infused with D-IIR or control IgG were further analyzed with a digitizer (Silicon Coach). The videos (60 frames per s) were viewed frame by frame, and at least 30 s of the hind paw position on day 3 of infusion was manually digitized for each mouse to track changes over time. The steps per min and skips per min (defined as step duration ≤500 ms) were counted visually and confirmed by digitization for a trial on day 3. Each mouse was also observed and filmed in a blinded fashion in open-field locomotion for 5 min per day, and the amount of time the mouse was ambulatory in 2.5-min blocks and the number of times each mouse stood up independently with full extension of the hind limbs (vertical stance) was determined by visual inspection.

Histological Analysis. At day 3 after surgery, the mice were anesthetized with isoflurane and underwent intracardiac perfusion with PBS containing heparin, 4% paraformaldehyde, and 0.1% glutaraldehyde. Brains were sectioned in parasagittal and coronal planes, processed routinely, and embedded in paraffin. Four-micron sections were stained in H&E and analyzed in a blinded fashion by a trained neuropathologist. All mice had enlargement of the third, lateral, and fourth ventricles, consistent with a communicating hydrocephalus, likely secondary to the amount and rate of protein infused. Purkinje cell soma, dendrite, and axons were studied by anti-calbindin monoclonal antibody (Sigma) with routine immunohistochemical methods and visualized by Zeiss confocal microscopy. Because Purkinje and granule cell degeneration is known to occur in patients with paraneoplastic cerebellar ataxia, we looked for evidence of cell death in infused mice. We focused on the inferior vermis, a region far from the implantation site but well infused with IgG. Slides were counterstained with hematoxylin and imaged with a Zeiss microscope with a 100 x objective, NA 1.7, n = 4 for D-IIIR mice and n = 3 for control mice. Evidence of any kind of cell death was also assessed by transmission electron microscopy. Coronal and parasagittal cerebellar sections from three experimental and three control mice were osmicated, serially dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Epon epoxy resin. Thick sections were stained with toluidine blue and examined with a Zeiss light microscope to select an area in the caudal vermis for ultrathin sectioning. Eighty-nanometer sections were placed on carbon/formvar-coated copper grids and stained with uranyl acetate and lead citrate, followed by viewing in a JEOL 1230 at 80 kV and photographed with a Gatan digital camera at 2–120,000×.

Statistical Analysis. A two-tailed Student’s t test was used to determine the level of significance. Repetitive ANOVA was used to calculate the significance of the Rota Rod test.

ACKNOWLEDGMENTS. C. Barrett and A. Kreitzer contributed to preliminary experiments. We thank Drs. T. Hwang, R. Malenka, J. Perrino, D. Profitt, D. Saal, M. Shiao, R. Tolwani, and members of the Tsien laboratory for invaluable assistance. This work was supported by a K08 grant from the National Institute of Neurological Disorders and Stroke/National Institutes of Health (NIH) and a Career Award in Biomedical Sciences from the Burroughs Wellcome Foundation (to Y.J.L.) and by NIH R01 grants (to R.W.T.).