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A Monoclonal Antibody to the β Subunit of the Skeletal Muscle Dihydropyridine Receptor Immunoprecipitates the Brain ω-Conotoxin GVIA Receptor*

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Junshi Sakamoto and Kevin P. Campbell‡

From the Howard Hughes Medical Institute, Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

Antibodies against the subunits of the dihydropyridine-sensitive L-type calcium channel of skeletal muscle were tested for their ability to immunoprecipitate the high affinity (K_d = 0.13 nM) ^{125}I-ω-conotoxin GVIA receptor from rabbit brain membranes. Monoclonal antibody VD2$_1$ against the β subunit of the dihydropyridine receptor from skeletal muscle specifically immunoprecipitated up to 86% of the ^{125}I-ω-conotoxin receptor solubilized from brain membranes whereas specific antibodies against the α_1, α_2, and γ subunits did not precipitate the brain receptor. Purified skeletal muscle dihydropyridine receptor inhibited the immunoprecipitation of the brain ω-conotoxin receptor by monoclonal antibody VD2$_1$. The dihydropyridine receptor from rabbit brain membranes was also precipitated by monoclonal antibody VD2$_1$. However, neither the neuronal ryanodine receptor nor the sodium channel was precipitated by monoclonal antibody VD2$_1$. The ω-conotoxin receptor immunoprecipitated by monoclonal antibody VD2$_1$ showed high affinity ^{125}I-ω-conotoxin binding, which was inhibited by unlabeled ω-conotoxin and by $CaCl_2$ but not by nitrendipine or by diltiazem. An antibody against the β subunit of the skeletal muscle dihydropyridine receptor stained 58- and 78-kDa proteins on immunoblot of the ω-conotoxin receptor, partially purified through heparin-agarose chromatography and VD2$_1$-Sepharose chromatography. These results suggest that the brain ω-conotoxin-sensitive calcium channel contains a component homologous to the β subunit of the dihydropyridine-sensitive calcium channel of skeletal muscle and brain.

Voltage-gated calcium channels play a major role in the regulation of intracellular calcium concentration in many cell types, including those of striated and smooth muscle and those of the nervous system (1–4). The dihydropyridine-sensitive calcium channel of skeletal muscle is essential in excitation-contraction coupling (5) and has been proposed to have a dual function as a calcium channel and as a voltage sensor for excitation-contraction coupling (6). The skeletal muscle dihydropyridine receptor has been purified and consists of four subunits: α_1 (170 kDa), α_2 (175 kDa, nonreduced; 150 kDa, reduced), β (52 kDa), and γ (32 kDa) (1). All four subunits have been recently cloned (7–10).

In neurons, voltage-gated calcium channels exist in several types (L, N, T, and P) which have different kinetic and pharmacological properties (11, 12). Dihydropyridines bind specifically to L-type channels and alter their channel activities whereas the inhibitory effect of ω-conotoxin GVIA (ω-CgTX)[1] is largely specific for N-type channels and possibly for some subpopulation(s) of L-type channels (2, 3). Neuronal calcium channels play crucial roles in calcium regulation, especially the N-type calcium channels, which may be responsible for triggering neurotransmitter release at synapses (4, 11). Although several antibodies are reported to react with the brain dihydropyridine receptor (13–16), no antibodies specific for the ω-CgTX receptor have been reported. In addition, neuronal calcium channels have not been purified, and their molecular structures and subunit compositions are still unknown.

Our laboratory has been involved in the production and characterization of antibodies against the skeletal muscle dihydropyridine receptor (17–19). Here we report that a monoclonal antibody (mAb) against the β subunit of the skeletal muscle dihydropyridine receptor immunoprecipitates both the brain dihydropyridine receptor and the brain ω-CgTX receptor, suggesting that these receptors have components homologous to the β subunit of the skeletal muscle dihydropyridine receptor.

EXPERIMENTAL PROCEDURES

Preparation of Rabbit Brain Membranes—Whole rabbit brains were homogenized in 50 mM Tris-HCl, pH 7.4, in the presence of the protease inhibitors aprotinin (76.8 nM), benzamidine (0.75 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (0.1 mM). The homogenate was centrifuged at 35,000 × *g* for 15 min. The pelleted membranes were resuspended in 0.3 M sucrose, 20 mM Tris maleate, pH 7.4, containing protease inhibitors, frozen in liquid nitrogen, and stored at −135 °C. Protein was determined by the method of Lowry *et al.* (20) as modified by Peterson (21) using bovine serum albumin as a standard.

^{125}I-ω-CgTX Binding Assay—Brain membranes (1 mg of protein/ml) were incubated with various concentrations of ^{125}I-ω-CgTX for 1 h at room temperature in buffer A (10 mM Hepes-NaOH, pH 7.4, 0.75 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.1 M NaCl and 0.2 mg/ml bovine serum albumin. The amount of ^{125}I-ω-CgTX bound was determined by filtration on Whatman GF/B filters as described previously (22) using buffer A containing 0.1 M NaCl and 1 mg/ml bovine serum albumin as the wash buffer. Specific

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‡ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be sent: Howard Hughes Medical Institute, University of Iowa College of Medicine, 400 Eckstein Medical Research Bldg., Iowa City, IA 52242.

[1] The abbreviations used are: ω-CgTX, ω-conotoxin GVIA; mAb, monoclonal antibody; GAM beads, goat anti-mouse IgG-Sepharose beads; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

binding was calculated by subtracting nonspecific binding determined in the presence of 0.5 μM unlabeled ω-CgTX from total binding. For the solubilized receptor the amount of bound ^{125}I-ω-CgTX was assayed with a rapid gel filtration method (23). Briefly, samples (0.2 ml) were layered on 1.5-ml columns of Sephadex G-50 (fine) preequilibrated with buffer A containing 0.1% digitonin and 0.1 M NaCl and centrifuged at 1,000 × g for 1 min in a swinging bucket rotor. The radioactivity in the void volume was counted with a γ-counter.

Immunoprecipitation of ^{125}I-ω-CgTX Receptor from Solubilized Brain Membranes—mAbs were coupled to goat anti-mouse IgG-Sepharose beads (GAM beads) as described previously (24). Polyclonal antisera or ascites fluid (5 μl) were coupled to 50-μl protein A-Sepharose beads by incubating overnight at 4 °C. The beads were washed twice in phosphate-buffered saline (50 mM sodium phosphate, pH 7.4, and 150 mM NaCl) by centrifugation and preequilibrated with buffer A containing 0.1% digitonin and 0.1 M NaCl. Rabbit brain membranes (2 mg of protein/ml) were labeled with 0.5 nM ^{125}I-ω-CgTX as described above. The labeled receptor was solubilized in buffer A with 1% digitonin and 1 M NaCl. After a 1-h incubation at 4 °C, samples were centrifuged at 100,000 × g for 30 min, and the supernatant was diluted 10-fold with buffer A. Diluted samples (1 ml) were incubated with antibody beads at 4 °C for 3 h. After centrifugation, the beads were washed three times in buffer A with 0.1% digitonin and 0.1 M NaCl, and the radioactivity bound to the beads was measured with a γ-counter. Specific binding was calculated as described above.

Immunoprecipitation of the Dihydropyridine Receptor, Ryanodine Receptor, and Sodium Channel—The dihydropyridine receptor of rabbit whole brain membranes was prelabeled with 10 nM [^{3}H]PN200-110 in buffer A containing 0.1 mM diltiazem and 0.1 M NaCl at 37 °C for 1 h and solubilized by incubating with 2.5% CHAPS plus 10 mg/ml phosphatidylcholine at 4 °C for 1 h. The ryanodine receptor was prelabeled with 5 nM [^{3}H]ryanodine in buffer A containing 10 mM ATP, 0.8 mM $CaCl_2$, and 1.5 M KCl at 37 °C for 1 h and solubilized with 2.5% CHAPS plus 10 mg/ml phosphatidylcholine as described previously (25). The sodium channel was solubilized in buffer A containing 2.5% Triton X-100, 2.5 mg/ml phosphatidylcholine, and 0.1 M KCl (26). After centrifugation at 100,000 × g for 30 min the receptor remaining in the supernatant was labeled using 10 nM [^{3}H] saxitoxin in buffer A containing 5 mM $CaCl_2$ and 0.1 M KCl, by incubation on ice for 1 h. Immunoprecipitation assays of these receptors were performed as described above, using appropriate wash buffers. Nonspecific binding of [^{3}H]PN200-110, [^{3}H]ryanodine, and [^{3}H]saxitoxin in the beads was determined in the presence of 10 μM nitrendipine, 2.5 μM ryanodine, and 2.5 μM saxitoxin, respectively, and subtracted from the total binding.

^{125}I-ω-CgTX Binding Assay on Receptor Immunoprecipitated with mAb VD2$_1$—Unlabeled rabbit brain membranes were solubilized with digitonin and immunoprecipitated with mAb VD2$_1$-GAM beads as described above. The resulting beads were incubated with various concentrations of ^{125}I-ω-CgTX in buffer A plus 0.1% digitonin and 0.02 M NaCl in the presence or absence of calcium channel ligands at 4 °C for 1 h. After washing three times, the amount of ^{125}I-ω-CgTX bound to the beads was measured in a γ-counter.

Partial Purification and Immunoblot Analysis of ω-Conotoxin Receptor—Monoclonal antibody VD2$_1$ was covalently coupled to cyanogen bromide-activated Sepharose as described previously (27). Rabbit brain membranes (4 mg of protein) prelabeled with ^{125}I-ω-CgTX were mixed with unlabeled rabbit brain membranes (1,500 mg of protein), solubilized by incubating with 1% digitonin at 5 mg of protein/ml, and diluted 3.3-fold. The solubilized material was applied to a 50-ml heparin-agarose column. The proteins eluted from the column with buffer A containing 0.6 M NaCl and 0.1% digitonin were applied to a 5-ml VD2$_1$-Sepharose column. After washing the column with 10 column volumes of the same buffer, the ω-conotoxin receptor was eluted with 50 mM sodium acetate buffer, pH 4.5, containing 0.6 M NaCl, 0.1% digitonin and with 50 mM glycine-HCl buffer, pH 2.5, containing the same constituents. Immunoblot analysis was performed as described previously (24).

Materials—The dihydropyridine receptor of rabbit skeletal muscle was purified as described previously (28). Monoclonal antibodies against the α_1 subunit (IG3, IIC12, IIF7, and IIID5) and the β subunit (VD2$_1$) and guinea pig polyclonal antibodies against the α_2 and γ subunit were produced as described (17–19). A polyclonal antibody to the β subunit was affinity purified from guinea pig polyclonal antiserum raised against the whole dihydropyridine receptor complex as described previously (19) using the β subunit of skeletal muscle dihydropyridine receptor immobilized on polyvinylidene difluoride membranes and was designated GP48β. ^{125}I-Labeled and unlabeled ω-CgTX were purchased from Amersham Corp. and Peninsula Laboratories, respectively. [^{3}H]PN200-110, [^{3}H]ryanodine, and [^{3}H]saxitoxin were from Du Pont-New England Nuclear. Digitonin was from Sigma and was purified as described (17). Goat anti-mouse IgG-Sepharose, wheat germ agglutinin-Sepharose, and CHAPS were from Cappel, Pharmacia LKB Biotechnology Inc., and Pierce, respectively. Heparin-agarose, protein A-Sepharose, and phosphatidylcholine were from Sigma. All other chemicals were of reagent grade.

RESULTS

Immunoprecipitation of the ω-CgTX Receptor with Antibodies against the Subunits of the Skeletal Muscle Dihydropyridine Receptor—Saturation binding analysis was performed on rabbit brain membranes to determine the appropriate concentration of ^{125}I-ω-CgTX to label the ω-CgTX receptor. From a Scatchard plot of ^{125}I-ω-CgTX binding, values for K_d and B_{max} were calculated to be 0.13 nM and 204 fmol/mg of protein, respectively. The K_d value is in agreement with other reports (29–32). Since 0.5 nM ^{125}I-ω-CgTX was nearly enough to saturate the high affinity binding site, this concentration of the ligand was used for labeling the receptor thereafter.

Various antibodies against the individual subunits of the skeletal muscle dihydropyridine receptor have been produced in our laboratory (17–19). To determine whether or not these antibodies cross-reacted with components of the brain ω-CgTX receptor, immunoprecipitation assays were performed using mAb-GAM beads and polyclonal antibody-protein A-Sepharose beads. Fig. 1 shows that the anti-β subunit mAb VD2$_1$ specifically immunoprecipitated a significant amount of the receptor (61.5% of the added receptor) whereas four anti-α_1 subunit mAbs, two anti-α_2, and two anti-γ subunit polyclonal antibodies did not react with the receptor. Fig. 1 also shows that heparin-agarose significantly precipitated the receptor (97.3% of the added receptor). To determine the maximal precipitation of the ω-CgTX receptor by VD2$_1$, various volumes of VD2$_1$ ascites fluid coupled to GAM beads were used in immunoprecipitation assays (Fig. 2). GAM beads alone did not precipitate detectable amounts of the ^{125}I-ω-CgTX receptor. As the amount of VD2$_1$ in the incubation mixture increased, the amount of precipitated ^{125}I-ω-CgTX receptor increased and reached maximum at 3 μl ascites fluid. The maximal amount of the precipitated receptor, calculated

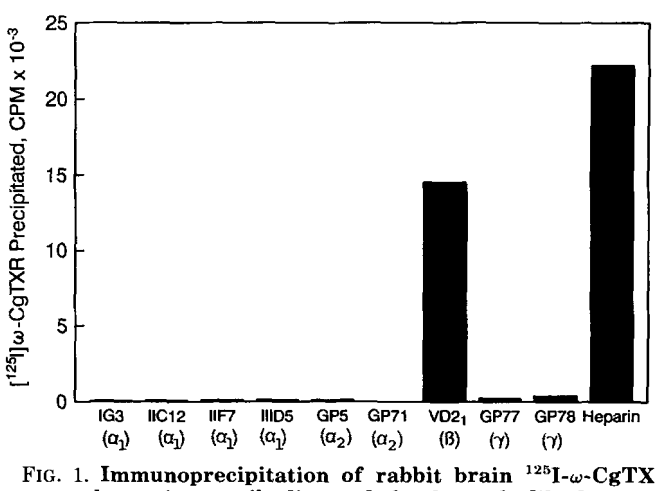

FIG. 1. **Immunoprecipitation of rabbit brain ^{125}I-ω-CgTX receptor by various antibodies to skeletal muscle dihydropyridine receptor.** Rabbit brain membranes were labeled with 0.5 nM ^{125}I-ω-CgTX and solubilized with digitonin. Antibody beads were formed as described under "Experimental Procedures." Precipitation assay was performed using 50 μl of antibody beads or heparin-agarose beads. *Parentheses* show the subunits of the skeletal muscle L-type calcium channel to which the antibodies are specific.

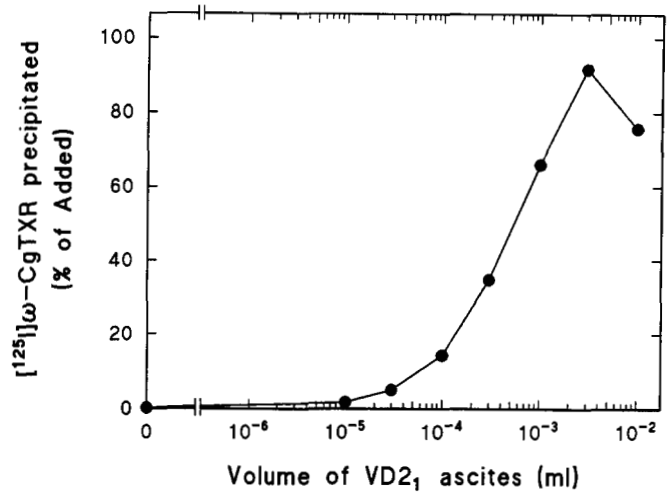

FIG. 2. **Dose dependence of $VD2_1$ immunoprecipitation of brain ^{125}I-ω-CgTX receptor.** Various volumes (0.01–10 μl) of mAb $VD2_1$ ascites fluid were incubated with GAM beads to form $VD2_1$-GAM beads. Immunoprecipitation was performed as in Fig. 1. The amounts of precipitated ^{125}I-ω-CgTX receptor were expressed as percentages of the amount of the added receptor, which was estimated with rapid gel filtration as described under "Experimental Procedures." The mean ± S.E. for the maximal percentage (85.9% ± 5.1%) was obtained from three separate experiments.

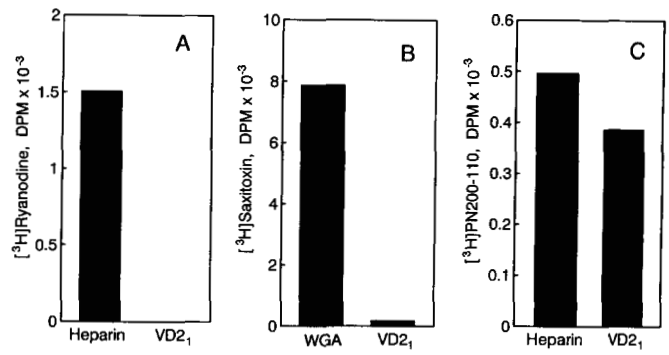

FIG. 3. **Precipitation of various receptors of rabbit brain by $VD2_1$.** The ryanodine receptor (*A*), the sodium channel (*B*), and the dihydropyridine receptor (*C*) of rabbit brain membranes were labeled with [^{3}H]ryanodine, [^{3}H]saxitoxin, and [^{3}H]PN200-110, respectively, and solubilized as described under "Experimental Procedures." These receptors were incubated with $VD2_1$-GAM beads, wheat germ agglutinin (*WGA*)-Sepharose, or heparin-agarose beads and assayed as described for Fig. 1.

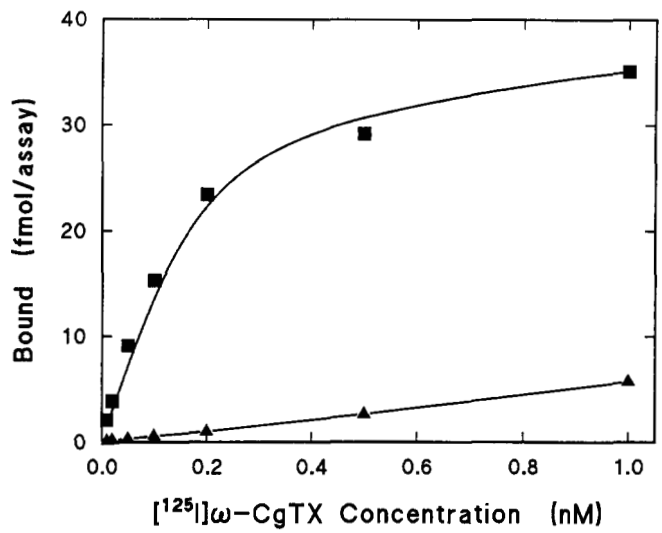

FIG. 4. **^{125}I-ω-CgTX binding to immunoprecipitate by $VD2_1$ from solubilized rabbit brain membranes.** The ω-CgTX receptor solubilized from rabbit brain membranes was immunoprecipitated by $VD2_1$-GAM beads as described under "Experimental Procedures." These beads were incubated with various concentrations of ^{125}I-ω-CgTX in the presence (▲) or absence (■) of 0.5 μM unlabeled ω-CgTX at 4 °C for 1 h. After washing three times, the amount of ^{125}I-ω-CgTX bound to the beads was measured.

from three separate experiments, was 85.9% ± 5.1% (mean ± S.E.) of the total amount of the receptor added to the incubation mixture.

Specificity of the Immunoprecipitation by mAb $VD2_1$—The dihydropyridine receptor purified from skeletal muscle was able to compete with the brain ^{125}I-ω-CgTX receptor for binding to mAb $VD2_1$. As the amount of purified dihydropyridine receptor added to the immunoprecipitation mixture increased, the amount of ^{125}I-ω-CgTX receptor precipitated by $VD2_1$ decreased (data not shown). When an excess of purified dihydropyridine receptor was added, the precipitated ^{125}I-ω-CgTX receptor was less than 20% of the control value. This finding indicates that mAb $VD2_1$ recognizes an epitope in the brain ω-CgTX receptor which is similar to that in the skeletal muscle dihydropyridine receptor.

The specificity of $VD2_1$ was investigated further by examining its ability to immunoprecipitate other neuronal membrane receptors. Neither the brain ryanodine receptor (Fig. 3*A*) nor the brain sodium channel (Fig. 3*B*) was immunoprecipitated by mAb $VD2_1$. Heparin-agarose and wheat germ agglutinin-Sepharose, respectively, were used as positive controls for these receptors since these affinity beads have been reported to bind the receptors (25, 33). Monoclonal antibody $VD2_1$ was found to immunoprecipitate the brain dihydropyridine receptor (Fig. 3*C*). The brain dihydropyridine receptor was analogous to the ω-CgTX receptor in having affinity for heparin-agarose. Thus, both the ω-CgTX receptor and the dihydropyridine receptor were precipitated by mAb $VD2_1$-GAM beads and heparin-agarose.

Characterization of the ω-CgTX Receptor Immunoprecipitated by mAb $VD2_1$—To establish that $VD2_1$ immunoprecipitates the high affinity ω-CgTX receptor, ^{125}I-ω-CgTX postlabeling experiments were performed on the receptor immunoprecipitated by $VD2_1$. Fig. 4 shows total and nonspecific binding to the immunoprecipitated ω-CgTX receptor. The K_d value was calculated to be 0.026 nM. Thus, it is clear that $VD2_1$ immunoprecipitated the high affinity ω-CgTX receptor. Fig. 5 shows the effects of calcium channel ligands on ^{125}I-ω-CgTX binding to the receptor immunoprecipitated by $VD2_1$. Unlabeled ω-CgTX inhibited ^{125}I-ω-CgTX binding to the immunoprecipitate whereas ligands to the L-type calcium channels, nitrendipine and diltiazem, did not affect ^{125}I-ω-CgTX binding. $CaCl_2$ added at a final concentration of 10 mM also inhibited ^{125}I-ω-CgTX binding to the receptor. These binding properties are similar to those reported for the high affinity ω-CgTX receptor in intact membranes from mammalian brain (29, 30).

Immunoblot Analysis of Partially Purified ω-CgTX Receptor—To identify the β subunit in isolated brain membranes, immunoblot analysis was performed. However, no staining was detected in whole rabbit brain membranes with mAb $VD2_1$, probably because of the low content of receptor in brain membranes. To increase the sensitivity of detection of the β subunit, the ω-CgTX receptor was partially purified. Brain membranes (1,500 mg) were prelabeled with ^{125}I-ω-CgTX, solubilized with digitonin, and applied to a heparin-agarose column. The eluate from this column was applied to a $VD2_1$-Sepharose column followed by elution with acidic buffers. The

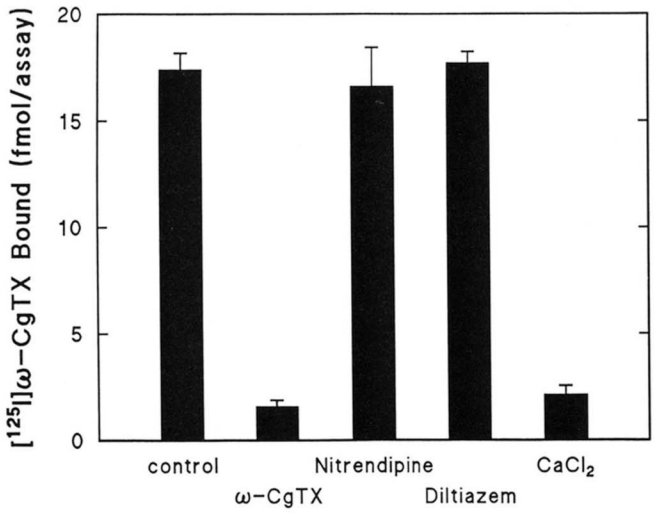

FIG. 5. **Effect of calcium blockers on ^{125}I-ω-CgTX binding to the VD2$_1$ immunoprecipitate.** The receptor immunoprecipitated with VD2$_1$-GAM beads was postlabeled with 0.5 nM ^{125}I-ω-CgTX in the presence or absence of 0.5 μM unlabeled ω-CgTX, 10 μM nitrendipine, 10 μM diltiazem, or 10 mM $CaCl_2$, and the amount of ^{125}I-ω-CgTX bound to the beads was measured as described under "Experimental Procedures."

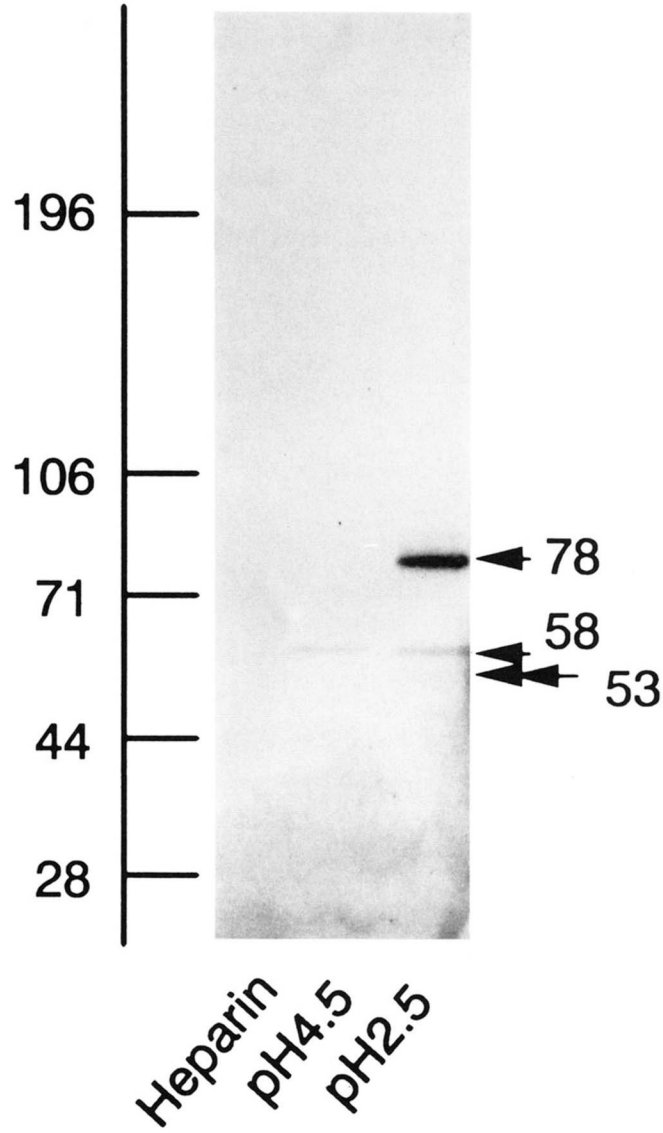

FIG. 6. **Immunoblot analysis of partially purified ω-CgTX receptor.** ω-CgTX receptor was partially purified through heparin-agarose chromatography and VD2$_1$-Sepharose chromatography as described under "Experimental Procedures." 100 μg of heparin-agarose eluate (*Heparin*) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Eluates (1.5 ml each) of VD2$_1$-Sepharose chromatography with pH 4.5 buffer (*pH 4.5*) and pH 2.5 buffer (*pH 2.5*) were concentrated with Centricon-30 microconcentrators and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were transferred to nitrocellulose membrane and immunoblotted with affinity-purified antibody GP48β to β subunit of skeletal muscle dihydropyridine receptor with 1:100 dilution. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left*. The *double arrowhead* indicates the position of the β subunit of the skeletal muscle dihydropyridine receptor.

overall recovery of ^{125}I-ω-CgTX in the pH 4.5 and 2.5 eluates was 12.7 and 5.6%, respectively, whereas the recovery of proteins was 0.8×10^{-3}% and 1×10^{-3}%, respectively. Since the eluted receptors were inactive in ω-CgTX binding, the enrichment of receptor was not accurately estimated. However, by assuming that most proteins bound to VD2$_1$-Sepharose were eluted under these harsh conditions, the overall enrichment of the receptor could be calculated to be 10,000-fold from these values. These eluates were analyzed by immunoblotting with guinea pig antibody GP48β affinity-purified against the β subunit of skeletal muscle dihydropyridine receptor. As shown in Fig. 6, the eluate from the VD2$_1$-Sepharose column with pH 2.5 buffer showed 58-kDa and 78-kDa proteins on the immunoblot whereas the eluate with pH 4.5 buffer showed only a 58-kDa protein. This staining was only seen with the affinity-purified anti-β subunit antibody and not seen with an affinity-purified anti-α_1 subunit antibody, an anti-α_2 subunit antibody, antisera from control guinea pigs, or with the secondary antibody alone (data not shown). Monoclonal antibody VD2$_1$ gave results similar to GP48β although the staining was weaker. These apparent molecular masses were clearly larger than that of the β subunit of the skeletal muscle dihydropyridine receptor (53 kDa). The two consecutive affinity chromatographies were useful for enriching the proteins reactive to the anti-β subunit antibodies; however, it should be noted that the eluate from the VD2$_1$-Sepharose column lost binding activity to ^{125}I-ω-CgTX, probably because of the acidic elution conditions.

DISCUSSION

We have tested the ability of various antibodies against the individual subunits of the dihydropyridine-sensitive L-type calcium channel from skeletal muscle to immunoprecipitate the ω-CgTX receptor from brain. An anti-β subunit mAb VD2$_1$ specifically immunoprecipitated the ω-CgTX receptor (Fig. 1). The dihydropyridine receptor purified from skeletal muscle inhibited the immunoprecipitation, and mAb VD2$_1$ did not immunoprecipitate the other neuronal ion channels (Fig. 3, *A* and *B*), indicating the specificity of this reaction. These results suggest that the brain ω-CgTX receptor contains a component homologous to the β subunit of the dihydropyridine receptor. Ruth *et al.* (9) reported that a cDNA encoding the β subunit of the skeletal muscle hybridizes with mRNA from brain under high stringency. By using a similar cDNA as a probe, a cDNA from brain has been isolated which is highly homologous but not identical with the skeletal muscle clone (34). These reports indicate that the brain contains a protein similar to the β subunit of the skeletal muscle dihydropyridine receptor and are consistent with the results of the present study utilizing antibodies.

Monoclonal antibody VD2$_1$ also immunoprecipitated the rabbit brain dihydropyridine receptor (Fig. 3*C*), suggesting

that the brain dihydropyridine receptor also has a component homologous to the β subunit of skeletal muscle dihydropyridine receptor. Thus, the relationship of the two brain receptors in the immunoprecipitate was examined. Calcium blockers such as nitrendipine (a dihydropyridine derivative) and diltiazem (a benzothiazepine), which both block L-type calcium channel, did not affect the ^{125}I-ω-CgTX binding to the immunoprecipitated receptor whereas unlabeled ω-CgTX and $CaCl_2$ inhibited the binding (Fig. 5). These binding properties are similar to those reported for the high affinity ω-CgTX receptor in intact membranes from mammalian brain (29, 30). ω-CgTX was initially suggested to block not only the N-type calcium channel but also the L-type calcium channel (35). However, recent electrophysiological data from several laboratories indicate that ω-CgTX does not block the L-type channel (36–38). Furthermore, several mAbs are reported to immunoprecipitate most of the brain dihydropyridine receptors but not more than 13% of the ω-CgTX receptors (14–16). These findings suggest that the majority if not all of the ω-CgTX receptor is distinct from the dihydropyridine receptor in brain. Our results, along with those reports, indicate that both of these receptors have a similar β subunit but have distinct components for ligand binding. Snutch *et al.* (39) recently described four classes of rat brain cDNAs which are homologous to that of the α_1 subunit of heart and skeletal muscle L-type calcium channels; however, it is not yet known if one of these cDNAs encodes a ω-CgTX binding component of the N-type channel.

The structural conservation of the β subunit over the different types of calcium channels suggests that this subunit plays an important role in calcium channel regulation. It has been shown that the β subunit of the skeletal muscle dihydropyridine receptor is phosphorylated by a protein kinase intrinsic to the isolated triads (40) and cyclic AMP-dependent protein kinase (40, 41) although its physiological role is not clear (42).

After enrichment of the ^{125}I-ω-CgTX receptor with heparin-agarose chromatography and $VD2_1$-Sepharose chromatography, 58- and 78-kDa proteins were detected with mAb $VD2_1$ and affinity-purified polyclonal antibody GP48β to the β subunit of the skeletal muscle dihydropyridine receptor. The apparent molecular weights of these bands were clearly larger than that of the β subunit of the skeletal muscle dihydropyridine receptor. This agrees with data from analysis of the brain and skeletal muscle cDNAs for the β subunit in which the brain cDNA predicts a protein of 5 kDa larger molecular mass than the skeletal muscle counterpart (34). In addition, both of these brain proteins were stained with an antiserum raised against a synthetic peptide with a sequence deduced from the brain cDNA for the β subunit.[2] Since the brain dihydropyridine receptor as well as the brain ω-CgTX receptor bind to both mAb $VD2_1$ and heparin-agarose (Figs. 1 and 3*C*), it is probable that both receptors are enriched in the partially purified sample. Therefore, the 58- and 78-kDa proteins could be the β subunits of these two receptors. Ahlijanian *et al.* (15) showed that mAbs to the $\alpha_2\delta$ subunits of the skeletal muscle L-type calcium channel immunoprecipitated the brain dihydropyridine receptor. The antibodies precipitate a 57-kDa protein band in addition to 175-, 142-, and 100-kDa proteins. Based on the similarity of its apparent molecular mass, it was suggested the 57-kDa protein could be homologous to the β subunit of skeletal muscle L-type calcium channels. More investigation is necessary before determining if the 57-kDa protein is identical to the 58-kDa protein detected in our partially purified sample.

[2] M. Pragnell and K. P. Campbell, unpublished data.

The K_d values for the ^{125}I-ω-CgTX binding to the isolated membranes and to the immunoprecipitated solubilized receptor were 0.13 and 0.026 nM (Fig. 4), respectively. The difference between the two values may be caused by solubilization of the receptor with digitonin as reported previously (43) and/or by the slight difference in the incubation conditions. These values are in agreement with previous reports (29–32) although the total range of the reported K_d values are very wide (~1–3,500 pM; 43, 44). We did not observe very high affinity ω-CgTX binding with a K_d of approximately 1 pM as reported by Barhanin *et al.* (44). However, our experiment does not exclude the possible contribution of a small proportion of binding with a picomolar K_d, as described previously (43, 45). Recently, Horne *et al.* (46) demonstrated that there are heterogenous low affinity ω-CgTX binding sites ($K_d \sim 1$ μM) in *Discopyge ommata* electric organ and that the majority of them are on the α-bungarotoxin-sensitive nicotinic acetylcholine receptor. However, we have not detected ^{125}I-α-bungarotoxin binding in the immunoprecipitates by $VD2_1$ from rabbit brain membranes (data not shown). Furthermore, the brain ω-CgTX receptor showed 4 orders of magnitude higher affinity to ω-CgTX than the acetylcholine receptor of the electric organ. Therefore, it is unlikely that the ω-CgTX receptor precipitated with mAb $VD2_1$ is related to the α-bungarotoxin-sensitive nicotinic achetylcholine receptor. Monoclonal antibody $VD2_1$ did not immunoprecipitate the ryanodine receptor or the sodium channel of rabbit brain (Fig. 3, *A* and *B*). This suggests that β subunit-like components are specific for voltage-gated calcium channels which form a family of structurally related members.

We have shown that mAb $VD2_1$ to the β subunit of the skeletal muscle dihydropyridine receptor immunoprecipitates the brain ω-CgTX receptor and the brain dihydropyridine receptor. This result suggests that the N-type and L-type calcium channels of brain contain a component homologous to the β subunit of skeletal muscle L-type calcium channel. In addition, mAb $VD2_1$ should be useful to purify and further characterize these channels.

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