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Isolation and characterization of a monoclonal antibody against the saxitoxin-binding component from the electric organ of the eel *Electrophorus electricus*

(sodium channel/tetrodotoxin binding/membrane protein)

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ABSTRACT **A monoclonal hybridoma cell line secreting antibody against the saxitoxin-binding component from the eel *Electrophorus electricus* has been isolated. The specificity of this monoclonal antibody was established by (*i*) its ability to immunoprecipitate bound [^{3}H]saxitoxin from a detergent extract of electroplax membranes in a dose-dependent manner, (*ii*) the inability of unrelated monoclonal antibodies to immunoprecipitate the toxin-binding activity in a similar assay, and (*iii*) the ability of excess unlabeled tetrodotoxin to displace [^{3}H]saxitoxin from the immunoprecipitated component. The antibody is of the subclass IgG1 and binds specifically to a polypeptide component of $M_r \approx$ 250,000 on NaDodSO$_4$/polyacrylamide gels. The antigenic determinant is associated with the same polypeptide component throughout the purification procedure, indicating that this component is not a result of artifactual aggregation or degradation during isolation. We conclude that the 250,000-dalton polypeptide is part of the saxitoxin binding/sodium channel protein in the native electroplax membrane.**

The conduction of electrical impulses in nerve and muscle is mediated by voltage-sensitive ion channels located in the cell membrane (1). Much of our current understanding of the voltage-dependent sodium channel at the molecular level has come from studies utilizing the potent neurotoxins tetrodotoxin (TTX) and saxitoxin (STX) which are known to bind with relatively high affinity to the channel (reviewed in ref. 2). Questions concerning the structure of the sodium channel protein(s) have been approached by purification of the TTX- or STX-binding component from the electric organ of the eel *Electrophorus electricus* (3, 4), from rat and bovine brain (5, 6), and from the sarcolemma of rat skeletal muscle (7). These purifications have produced preparations with a specific toxin-binding activity no greater than 30–50% of that expected for homogeneous sodium channel [assuming one toxin molecule bound per channel molecule of 230,000 daltons (3)]. Because these preparations are only partially purified, it has been difficult to identify unambiguously the polypeptides that comprise the sodium channel. Agnew *et al.* (4) proposed that a 250,000-dalton component is part of the eel channel, and Hartshorne and Catterall (5) obtained partially purified material from rat brain containing protein components of M_r 270,000 and 38,000, in agreement with their previous results with photoactivatable derivatives of scorpion toxin (8). Barchi *et al.* (7) suggested that proteins of M_r 64,000, 60,000, and 54,000 are constituents of the rat sarcolemma channel, and Hucho *et al.* (9), studying a photoactivatable derivative of sea anemone toxin, proposed that a 53,000-dalton protein is part of the channel from crayfish nerve. Assignment of these polypeptides to the sodium channel, however, must be considered tentative.

Monoclonal antibodies directed against the sodium channel should be useful for investigating the molecular structure and properties of the channel. First, they could be used to identify decisively the polypeptides that are part of the channel complex. Second, they may be useful in defining what parts of the channel complex are involved in various functions—e.g., ion translocation, gating, and inactivation. Monoclonal antibodies would also be particularly useful for studying regulation of the synthesis, appearance, and localization of sodium channels.

In this report we describe the isolation of a monoclonal hybridoma cell line that secretes antibody directed against the STX-binding component from the electroplax of *E. electricus*. This antibody binds to a polypeptide of $M_r \approx$ 250,000 (referred to herein as p250).

MATERIALS AND METHODS

Materials. Electric organs from medium-sized *E. electricus* (World Wide Scientific Animals, Ardsley, NY) were frozen in liquid nitrogen immediately after dissection and stored at −90°C until used. [^{3}H]TTX (0.2 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and deionized Lubrol-PX (Sigma) were prepared as described (3). L-α-Phosphatidylcholine (type V-E from egg) and chromatographic media were purchased from Sigma.

NS1/SP2 myeloma cells were the generous gift of K. Barald. Polyethylene glycol was obtained from Dow Chemicals and RPMI-1640 culture medium, glutamine, penicillin, and streptomycin were from GIBCO. Thymidine, hypoxanthine, and aminopterin were from Sigma. Calf sera were purchased from GIBCO and screened for the ability to support hybridoma growth at limiting dilution. Purified mouse IgG was obtained from Miles and Pel-Freeze, and used for production of rabbit anti-mouse IgG antisera. Lyophilized goat anti-rabbit IgG and sheep anti-mouse IgG antisera were obtained from Cappel Laboratories (Cochranville, PA). Prior to use in solid-phase radioimmunoassays (RIAs), immunoglobulin was purified from these antisera by affinity chromatography on Sepharose coupled to rabbit or mouse IgG. Purified *Staphylococcus aureus* protein A was from Pharmacia. Radioiodinated protein A and goat anti-rabbit IgG immunoglobulin were prepared by the chloramine-T method. STX (provided by the National Institutes of Health) was radiolabeled by exchange with carrier-free tritiated water (10). The labeled reaction mixture was lyophilized and purified

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Abbreviations: RIA, radioimmunoassay; STX, saxitoxin; TTX, tetrodotoxin; P_i/NaCl, phosphate-buffered saline (0.05 M sodium phosphate, pH 7.4/0.9% NaCl/0.1% NaN_3).

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by high-voltage paper electrophoresis. The resulting toxin had a specific activity of 5.4 Ci/mmol and a radiochemical purity of >85% as determined by bioassay using frog sciatic nerve.

Antigen Purification. The TTX-binding component was purified from *E. electricus* electroplax essentially as described by Agnew *et al.* (3). In brief, electroplax membrane fragments were prepared by homogenizing the organ in 50 mM potassium phosphate, pH 7.4/5 mM phenylmethylsulfonyl fluoride/5 mM EDTA/5 mM iodoacetamide, filtering through gauze sieves, and centrifuging. The membrane preparation was solubilized with 1% Lubrol-PX and centrifuged at 100,000 × *g* for 1 hr to yield the detergent extract. The TTX-binding component was further purified by DEAE-Sephadex ion exchange chromatography (yielding the DEAE fraction), followed by gel filtration on a Sepharose 6B column. The TTX-binding peak was then rechromatographed on Sepharose 6B to yield the Sepharose 6B fraction. The specific activities of the successively more purified fractions from a typical preparation were 8–10, 10–15, 150–250, and 700–1100 pmol of TTX binding sites per mg of protein for the membranes, detergent extract, DEAE fraction, and Sepharose 6B fraction, respectively.

Immunization, Cell Fusion, and Cloning. One-month-old female BALB/cJ mice (Jackson Laboratory) were immunized with 400–500 μg of Sepharose 6B fraction emulsified in Freund's complete adjuvant (day 0). On days 65, 88, and 106, the mice were given 200–400 μg of the Sepharose 6B fraction emulsified in Freund's incomplete adjuvant. TTX-binding component constituted approximately 20–33% of the Sepharose 6B fraction. Three days after the final injection, 2.0×10^8 spleen cells and 5×10^7 NS1 myeloma cells were fused in the presence of polyethylene glycol and plated in hypoxanthine/aminopterin/thymidine selective medium (11). Hybrid cells were passaged and grown in RPMI-1640 medium supplemented with 20% calf serum, 2 mM glutamine, 1 mM pyruvate, 0.5 mM oxaloacetic acid, and 1 mg of penicillin/streptomycin per ml. Secretion of immunoglobulins was detected by an enzyme-linked immunosorbent assay (12). Monoclonal hybridoma cell lines were established by two successive clonings at limiting dilutions in 96-well plates, first at 0.2 cell per well and then at 0.1 cell per well. Ascites fluids were produced by injecting 1–3 × 10^6 monoclonal hybridoma cells intraperitoneally into pristane-primed BALB/c mice.

NaDodSO$_4$/Polyacrylamide Gel Purification of p250. Sepharose 6B fraction (3–10 mg) was electrophoresed on a preparative 6% NaDodSO$_4$/polyacrylamide gel (13) in the presence of reducing agent. After brief staining with Coomassie blue, the p250 band was cut from the gel and electroeluted. p250 was referred to as "band 1" by Agnew *et al.* (4). The resulting material was extensively dialyzed against 0.02% NaDodSO$_4$ and lyophilized.

Generation of Rabbit Antisera Against p250. Two female New Zealand White rabbits were immunized subcutaneously at multiple sites with 50–100 μg of gel-purified p250 (see above) in Freund's complete adjuvant and on day 84 were given a further 50–100 μg in Freund's incomplete adjuvant. The rabbits were bled on days 91, 95, and 99. These antisera were capable of precipitating the TTX-binding component from a detergent extract as measured by a rapid gel filtration assay for TTX binding (14).

Sepharose 6B RIA. Binding of antibody to the Sepharose 6B fraction was measured in a solid phase RIA. Microtiter wells were coated for 2 hr at 37°C with 20–40 ng of Sepharose 6B fraction in 30 μl of phosphate-buffered saline (P$_i$/NaCl; 0.05 M sodium phosphate, pH 7.4, 0.9% NaCl, 0.1% NaN$_3$). The Lubrol-PX concentration in this dilute Sepharose 6B fraction was <0.001%. Wells were then incubated with 30 μl of hybridoma supernatant overnight at 4°C and with 30 μl of rabbit anti-mouse IgG [(4 μg/ml) in P$_i$/NaCl containing 0.05% Tween 20, bovine serum albumin (Sigma, essentially globulin free, 1 mg/ml) and 0.1% Triton X-100] for 2 hr at room temperature. Wells were finally treated with ^{125}I-labeled protein A (100,000 cpm/well; 40 cpm/pg) in 30 μl of P$_i$/NaCl containing 0.05% Tween 20 and 0.25% gelatin for 2 hr at room temperature. Between steps, wells were washed three times with 100 μl of P$_i$/NaCl containing 0.05% Tween 20. Radioactivity bound to the plates was removed by incubating wells with 0.1 ml of 2 M NaOH for 5–15 min at room temperature and measured in a gamma counter.

Sandwich RIA. Wells of a 96-well microtiter plate were coated with 30 μl of affinity-purified sheep anti-mouse IgG (3.3 μg/ml in P$_i$/NaCl) for 2 hr at 37°C. Wells were subsequently treated with: 30 μl of hybridoma supernatant overnight at 4°C; 1 μg of Sepharose 6B fraction in 30 μl of P$_i$/NaCl/Tween/albumin/Triton for 2 hr at room temperature; 30 μl of rabbit antiserum to the electroeluted p250 (diluted 1:500 in P$_i$/NaCl/Tween/albumin/Triton) for 2 hr at room temperature; and 30 μl of ^{125}I-labeled goat anti-rabbit IgG (100,000 cpm/well; 50 cpm/pg) in P$_i$/NaCl/Tween/gelatin for 2 hr at room temperature. Between steps, wells were washed three times with 100 μl of P$_i$/NaCl/Tween. Bound radioactivity was removed and measured as in the Sepharose 6B RIA.

p250 RIA. Wells of a 96-well microtiter plate were coated with 60 ng of gel-purified electroeluted p250 in 30 μl of P$_i$/NaCl for 2 hr at 37°C. Wells were then allowed to react with hybridoma supernatant, rabbit anti-mouse IgG, and ^{125}I-labeled protein A as in the Sepharose 6B RIA.

Immunoprecipitation Assay. Partially purified or crude detergent extracts of sodium channel preparations from *E. electricus* (containing 15–75 pmol of TTX binding sites per ml) were incubated with 0.45 μM [^{3}H]STX in the presence or absence of an excess (50 μM) of unlabeled TTX at 0°C for 30 min. To 300 μl of this mixture was added 700 μl of culture supernatant or 0–25 μl of ascites fluid (supplemented with 8 mM EDTA) from a hybridoma line, and the incubation was continued for 7 hr at 0°C. Rabbit anti-mouse IgG antiserum (0.2 and 1.0 ml for the culture supernatant and ascites fluid, respectively) was then added and the mixture was incubated for 8 hr at 0°C. To achieve equivalence the appropriate amount of normal mouse IgG was added to the reaction, and incubation was continued for 2 hr at 0°C. Unbound [^{3}H]STX and [^{3}H]STX bound to uncomplexed sodium channels were separated from the immunoprecipitate by layering 1 ml of the reaction mixture over 300 μl of 30% (vol/vol) glycerol in P$_i$/NaCl in a 1.5 ml Eppendorf Microfuge tube. After 2-min centrifugation at 4°C, the tube was immediately frozen in liquid nitrogen. The portion of the tube containing the pellet was removed with a hot razor blade and the immune complex was dissolved in 400 μl of glacial acetic acid prior to scintillation counting in Aquasol. This pelleting procedure reduced the assay background to an acceptable level.

Antibody Staining of Polyacrylamide Gels. Electrophoresis was performed under nonreducing conditions in 6% polyacrylamide gels containing NaDodSO$_4$ (13). Gels were fixed, equilibrated with aqueous buffers, and treated sequentially with ascites fluids from hybridomas (1:2000 dilution), rabbit anti-mouse IgG (4 μg/ml of an affinity-purified preparation), and ^{125}I-labeled protein A (3–5 × 10^6 cpm; 40 cpm/pg) as described by Burridge (15).

RESULTS

Four BALB/c mice were primed and boosted with the Sepharose 6B fraction purified from *E. electricus*. This preparation was estimated to be approximately 25% sodium channel, as measured by specific TTX binding per mg of protein. Serum

from each of the four immunized mice was able to immunoprecipitate [^{3}H]TTX-binding activity from a detergent extract of electroplax membranes, indicating that all of the animals were responding to the sodium channel antigen (data not shown). After the final boosting injection, the spleen from one mouse was removed for fusion with NS1 myeloma cells. Viable hybrid cells were found in 242 of 251 wells initially plated.

Screening of Hybridomas. Three successive screening assays were performed on hybridoma supernatants in order to identify anti-sodium channel antibodies. First, the supernatants were tested for their ability to bind to Sepharose 6B fraction which was the antigen used in the immunization. This is not a rigorous test of specificity because of the impurity of this material. Second, the resulting positive clones were tested in the sandwich RIA for the ability to recognize components in the Sepharose 6B material that were simultaneously recognized by a rabbit antiserum against gel-purified p250. Agnew *et al.* (4) had shown that p250 copurifies with TTX-binding activity. Clones that were positive in this sandwich assay were thus producing antibody directed against either p250 or against some component of a larger complex that included p250. The rabbit antiserum against p250 clearly contained anti-sodium channel antibodies because it could precipitate the TTX-binding component from detergent extracts of electroplax membranes (data not shown). The ability of the sandwich RIA to identify monoclonal anti-sodium channel antibody unambiguously was limited, however, by the purity of p250 and the specificity of the resulting rabbit antiserum, neither of which has been rigorously established. Thus, for the third and definitive assay, clones that were positive in the sandwich RIA were grown in the larger quantitites necessary for testing in the immunoprecipitation assay. This assay identified monoclonal antibodies that were capable of precipitating [^{3}H]STX-binding activity from solubilized sodium channel preparations.

Fig. 1 shows the results from the three assays run on nine clones. These data were chosen to illustrate the elimination of clones and the selection of clone VD10 for further study. Of the initial 242 wells that contained hybridomas, 80 were scored as positive in the Sepharose 6B RIA. Of these 80, the 20 most positive were assayed by the sandwich RIA in which 5 clones were judged to be positive. These clones were then tested by the precipitation assay in which one clone designated VD10 was clearly positive. VD10 antibody was shown to be of the IgG1 subclass by Ouchterlony double-diffusion against subclass-specific rabbit antisera.

Characterization of Clone VD10. The relationship between VD10 antibody concentration and precipitation of the STX-binding component was analyzed in the immunoprecipitation assay. Fig. 2 shows the amount of radioactivity precipitated as a function of ascites fluid added. Because of the large volumes of rabbit anti-mouse IgG antiserum required to form the immune complexes, the dose–response curve could not be extended to a clear plateau under our experimental conditions. The highest level reached corresponded to precipitation of approximately 25% of the total [^{3}H]STX-binding activity present in the assay mixture. These data are consistent with a K_d for antibody–antigen reaction on the order of 10^{-7} M. The radioactivity precipitated was not due to precipitation of unbound [^{3}H]STX because the signal was abolished when the Sepharose 6B fraction was eliminated from the assay mixture (data not shown). The precipitated [^{3}H]STX was bound to specific toxin-binding sites because immunoprecipitation carried out in the presence of excess unlabeled TTX gave a low background that was invariant with the amount of ascites added. Under identical immunoprecipitation conditions, ascites fluid containing monoclonal IgG1 antibodies directed against an unrelated bovine pituitary antigen (16) did not precipitate significant radioactivity above background, either in the absence or presence of excess unlabeled TTX.

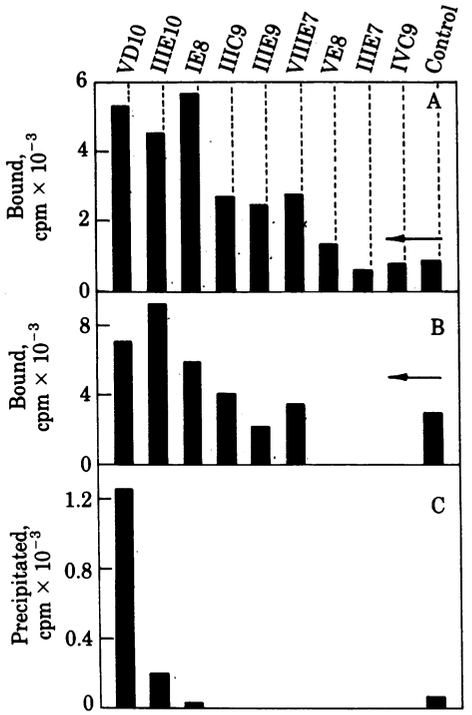

FIG. 1. Screening procedures used to identify anti-sodium channel antibody. Results from nine clones are shown; these clones were chosen to illustrate the identification of clone VD10 as an anti-sodium channel hybridoma. (*A*) Sepharose 6B RIA; (*B*) sandwich RIA; (*C*) immunoprecipitation assay. ^{125}I bound to the wells (*A* and *B*) or ^{3}H precipitated (*C*) is shown for each of the clones indicated. Control experiments for each assay were performed by replacing the hybridoma supernatant with RPMI medium plus supplements (*A* and *B*) or an IgG1 monoclonal antibody to an unrelated pituitary antigen (*C*) (16). The arrows in *A* and *B* indicate the cutoff levels for positive clones.

Identification of the Polypeptide Recognized by VD10 Antibody. To identify the polypeptide component(s) recognized

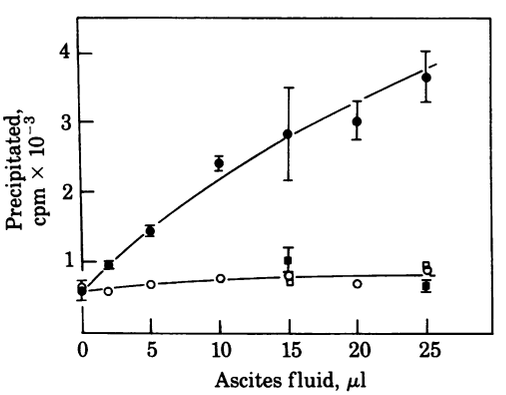

FIG. 2. Immunoprecipitation of the [^{3}H]STX binding component by VD10 antibody. A crude detergent extract of electroplax membranes was saturated with 450 nM [^{3}H]STX and incubated with varying concentrations of VD10 ascites fluid. The total amount of antibody in each tube was kept constant by supplementing with purified normal mouse immunoglobulin. Immune complexes were precipitated and the radioactivity associated with the pellet was plotted as a function of the volume of ascites fluid added. ●, VD10 in the absence of unlabeled TTX; ○, VD10 in the presence of 50 μM unlabeled TTX; ■, control antibody in the absence of TTX; □, same control antibody in the presence of 50 μM TTX. Error bars show SD about the mean of triplicate points.

by the VD10 antibody, sodium channel preparations from the four stages of purification (membranes, detergent extract, DEAE fraction, and Sepharose 6B fraction) were fractionated on 6% $NaDodSO_4$/polyacrylamide gels. These gels were then sequentially treated with VD10 antibody, rabbit anti-mouse IgG, and ^{125}I-labeled protein A (15). At each purification step, VD10 antibody reacted with a diffuse band of $M_r \approx 250,000$ (the p250 band) (Fig. 3). This band was not stained by an unrelated control IgG1 monoclonal antibody. The lane containing crude membranes also showed some reactivity at $M_r \approx 210,000$; this reactivity is nonspecific because it was present in gels treated with control monoclonal antibodies. None of the other numerous polypeptide components present in the various fractions reacted detectably with VD10 even on overloaded gels (e.g., Fig. 3, lanes 1). There was some diffuse staining in the M_r range 100,000–200,000 at the more advanced stages of purification (DEAE and Sepharose 6B fractions). This is likely to be due to degradation of the p250 which occurred during purification. Essentially identical staining patterns were observed under reducing and nonreducing conditions.

A RIA was performed in order to confirm that the p250 contained the antigenic determinant recognized by VD10 antibody. Microtiter plates were coated with gel-purified p250 and treated with varying amounts of VD10 antibody followed by rabbit anti-mouse IgG and ^{125}I-labeled protein A. Fig. 4 shows a binding curve for this assay. Control antibodies did not bind to the coated wells even at high concentrations, nor did VD10 antibody bind at detectable levels to wells coated with another membrane protein, the P_o glycoprotein of peripheral myelin (17).

DISCUSSION

The results indicate that our screening procedures were successful in identifying VD10 antibody as reacting with a component of the STX-binding complex. Several aspects of these procedures merit comment. Our ultimate criterion for specificity was the ability of the antibody to precipitate STX-binding activity from a detergent extract of electroplax membranes. It was not feasible to assay all 242 clones in this way because the specific activity of the [^{3}H]STX necessitated the use of large volumes of supernatant. Two RIAs (Sepharose 6B and sandwich) were therefore performed to decrease the number of clones to be assayed by the precipitation test. These in themselves were not sufficient to prove the specificity of an antibody, as discussed above. Note also that the p250 RIA was not used as part of the screening procedure because this assay would only detect antibodies that react with protein previously denatured with $NaDodSO_4$. In principle, our procedures permitted detection of antibodies that might only recognize native protein, although VD10 is apparently not in this category.

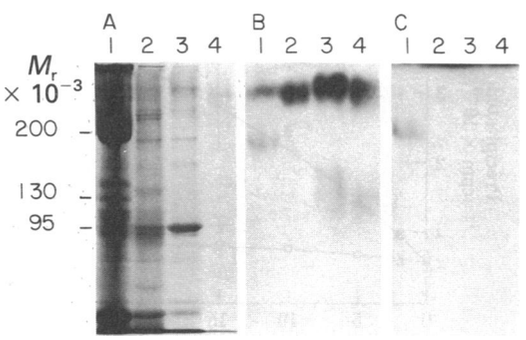

FIG. 3. Antibody staining of $NaDodSO_4$/polyacrylamide gels. Material from each of the four purification steps of the sodium channel preparation were analyzed on 6% $NaDodSO_4$/polyacrylamide gels under nonreducing conditions. The gels were stained with antibodies and ^{125}I-labeled protein A. Lanes 1, 2, 3, and 4 represent membranes, Lubrol extract, DEAE fraction, and Sepharose 6B fraction; respectively. Samples were boiled in 62.5 mM Tris, pH 6.8/3–6% $NaDodSO_4$/10% (vol/vol) glycerol immediately after purification and stored at −90°C until gel electrophoresis. (*A*) Coomassie blue staining of the gels. (*B*) Autoradiogram of a gel stained with VD10 antibody. (*C*) Autoradiogram of an identical gel stained with a control IgG1 monoclonal antibody (16).

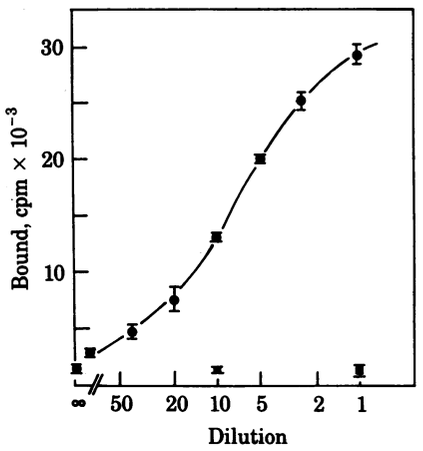

FIG. 4. Solid-phase RIA of VD10 antibody binding to gel-purified p250. Multiwell plates were coated with electroeluted p250 and incubated with varying dilutions of VD10 or control hybridoma supernatant. Binding was quantitated by reaction with rabbit anti-mouse IgG and ^{125}I-labeled protein A and plotted as a function of supernatant dilution. ●, Binding of VD10 antibody to p250; ■, binding of a control antibody in the same assay. Error bars show SD about the mean of triplicate points.

Several considerations indicate the specificity of the STX precipitation assay for anti-sodium channel antibody. The precipitation of radioactivity was abolished either by including excess unlabeled TTX or by omitting detergent extract from the assay mixture. Furthermore, antibodies against unrelated bovine and rat antigens were unable to precipitate radioactivity in this assay. Our results also indicate that the VD10 antibody is not simply recognizing a common antigenic determinant present on many membrane proteins. The pattern of antibody reactivity with crude electroplax membranes fractionated on $NaDodSO_4$ gels (Fig. 3) shows specific labeling of p250 only.

Previous work has shown that a band of $M_r \approx 250,000$ from purified preparations of eel electroplax membranes comigrates with TTX-binding activity after three independent fractionation methods (4). However, the composition of this high molecular weight band is subject to question. Although this material is visualized as a diffuse band in Coomassie blue-stained gels of purified preparations, it was possible that it arose by aggregation of lower molecular weight components during purification. Various reports (see *Introduction*) have suggested that lower molecular weight proteins are constituents of the sodium channel from some sources. Our results strongly suggest that p250 from eel does not arise by aggregation during purification. Fig. 3 demonstrates that the p250 recognized by VD10 antibody is present not only in purified preparations but also in crude membranes. No specific staining of lower molecular weight proteins is observed in the crude membrane lane. It remains to be seen whether lower molecular weight proteins not recognized by VD10 antibody are constituents of the eel sodium channel. Nonetheless, our results clearly implicate p250 as a component of the eel channel in the native membrane.

Note Added in Proof. Clone VD10 is available from the NIAID Hybridoma Bank administered by the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

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