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IMMUNOCHEMICAL PROPERTIES AND CYTOCHEMICAL LOCALIZATION OF THE VOLTAGE-SENSITIVE SODIUM CHANNEL FROM THE ELECTROPLAX OF THE EEL (*ELECTROPHORUS ELECTRICUS*)[1]

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Abstract

Immunochemical methods have been used to investigate questions concerning the relatedness of sodium channels from different sources and their distribution in the eel electroplax. The reagents employed were two monoclonal antibodies , 5D10 (Moore, H. -P. H., L. C. Fritz, M. A. Raftery, and J. P. Brockes (1982) *Proc. Natl. Acad. Sci. U. S. A. 79:* 1673–1677) and 5F3, and a rabbit antiserum; all three were directed against determinants present on the 250,000-dalton component of the eel sodium channel. In quantitative adsorption assays, the three reagents were effectively adsorbed by eel electroplax membranes but not by brain membranes from rat, frog, or chick. The rabbit antiserum bound to immobilized membranes of rat brain at a level only ~0.1% of that seen with electroplax membranes.

The reactivity of the three reagents with the eel electroplax was further investigated by indirect immunofluorescence on frozen sections. Whereas 5D10 showed no detectable reactivity, the rabbit antiserum and, especially, 5F3 stained the electrically excitable caudal face of the electrocytes but not the inexcitable rostral face. The reactivity of 5F3 was examined in greater detail and showed occasional abrupt discontinuities where the membrane was not stained. The presence of positive 5F3 immunoreactivity appeared to be correlated with extracellular filamentous material.

Voltage-sensitive sodium channels are responsible for the rapid upstroke of the action potential in excitable cells of many organisms (Hodgkin, 1964), and it appears that their physiological properties are highly conserved. Thus, channels from diverse sources behave similarly with regard to activation, inactivation, and ion selectivity (Hille, 1970). Recent biochemical evidence has further emphasized the similarities among sodium channels from different sources. Channel proteins have been partially purified from eel electroplax (Agnew et al., 1978), rat brain synaptosomes (Hartshorne and Catterall, 1981), and rat sarcolemma (Barchi et al., 1980; Weigele and Barchi, 1982), and in each case a high molecular weight glycoprotein has been observed to co-purify with the peak of binding of the specific channel toxins tetrodotoxin (TTX) and saxitoxin (STX). There may, however, also be significant differences among these channels. Current evidence suggests, for example, that different channel complexes can have distinct affinities for TTX and STX (Harris and Thesleff, 1971; Cohen et al., 1981; Lawrence and Catterall, 1981). Furthermore, differences may exist in the subunit composition of different sodium channels. The most purified channel preparations from rat brain synaptosomes contain polypeptides of M_r ~37,000 and 39,000 in addition to the large (M_r ~270,000) glycoprotein (Catterall, 1982). Preliminary evidence suggests that channels from rat sarcolemma also contain lower molecular weight components (Weigele and Barchi, 1982). Studies on eel electroplax channel, however, have provided clear evidence only for one component of M_r ~250,000 (Moore et al., 1982; Nakayama et al., 1982; Miller et al., 1983), although the exist-

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ence of lower molecular weight components cannot be totally ruled out (Nakayama et al., 1982).

The distribution of sodium channels in excitable cells is highly regulated. Physiological and pharmacological evidence from a number of sources suggests that in many cells the channels are not homogeneously distributed but, rather, are localized to particular regions of the plasmalemma (Waxman and Foster, 1980). In myelinated axons, for example, sodium channels are thought to be present exclusively in the unensheathed axolemma at the node of Ranvier (Ritchie and Rogart, 1977; Chiu and Ritchie, 1982). Electrophysiological recording from the eel electrocyte has shown that only one face of this flattened cell can generate an action potential, suggesting a difference in the density of channels in the two faces (Keynes and Martins-Ferreira, 1953).

In the present paper, immunological methods are used to investigate several aspects of sodium channel structure and distribution. The following questions are addressed: (*1*) How similar immunologically are sodium channels from various sources? (*2*) Are the antigenic determinants that are present in the channel exposed in the membrane-embedded state? (*3*) Can immunological reagents be used to visualize the sodium channel distribution in the eel electroplax?

To investigate these issues, the binding properties of three antibodies to the eel electroplax sodium channel have been studied. These reagents include monoclonal antibody (mAb) 5D10, some of whose properties have been described previously (Moore et al., 1982), mAb 5F3, described here for the first time, and a rabbit antiserum generated against the 250,000-dalton component (p250) from the eel sodium channel (Moore et al., 1982). Antibody 5F3 was generated in the same fusion that produced mAb 5D10 and has been shown to be of subclass IgG1 by Ochterlony double diffusion against subclass-specific antisera. Some of this work has been presented in preliminary form (Fritz and Brockes, 1983).

Materials and Methods

Electric organs from *Electrophorus electricus* were obtained from World Wide Scientific Animals (Ardsley, NY) and used for sodium channel purification as previously described (Agnew et al., 1978). Pieces of the organ of Sachs from *E. electricus* which were used for immunocytochemistry were obtained from Drs. M. Krouse and H. Lester, California Institute of Technology. Protocols for the preparation of [^{3}H]TTX and [^{3}H]STX, hybridoma production, anti-p250 antiserum production, and the Sepharose 6B radioimmunoassay (RIA) have been described previously (Moore et al., 1982). TTX-binding assays were performed as described by Agnew et al. (1978). Immunoprecipitation of the STX-binding component was performed as previously described (Moore et al., 1982) except that 3.5% polyethylene glycol (8000, Sigma Chemical Co., St. Louis, MO) was added 2 hr before centrifugation of the immune complexes.

Immunochemical methods. Western blots were prepared by separating electroplax proteins under non-reducing conditions by SDS-gel electrophoresis (Laemmli, 1970) in a linear polyacrylamide gradient of 4 to 12%. Proteins were transferred electrophoretically to nitrocellulose paper in a buffer of 190 mM glycine, 25 mM Tris, pH 8.3, containing 20% methanol or in 10 mM sodium borate (Towbin et al., 1979). The paper was blocked with either 0.25% gelatin or 5% hemoglobin for at least 1 hr and then reacted sequentially with hybridoma supernatant (diluted 1:2), affinity-purified rabbit anti-mouse IgG (4 μg/ml), and ^{125}I-protein A (3 to 5 $\times$ 10^6 cpm; 40 cpm/pg). One percent hemoglobin was present in each incubation. Western blots with rabbit anti-p250 antiserum were performed similarly except that the primary antibody incubation was with a 1:1000 dilution of antiserum followed by ^{125}I-protein A.

For adsorption assays, crude synaptosomal membrane preparations from brain were prepared as follows. Tissue was homogenized in 10 vol of 0.32 M sucrose, 5 mM sodium phosphate, pH 7.4, plus 0.1 mM phenylmethylsulfonyl fluoride, using a glass-Teflon homogenizer, and centrifuged at 1,000 $\times$ *g* for 10 min. The supernatant was saved, and the pellet was resuspended in the same buffer and centrifuged at 1,000 $\times$ *g* for 10 min. This supernatant was pooled with the saved supernatant and spun at 18,000 $\times$ *g* for 60 min. Pellets were resuspended in 50 mM potassium phosphate, pH 7.4, or 100 mM choline chloride, 20 mM HEPES, pH 7.4, and were stored in aliquots at −70°C. Rat liver and kidney membranes were prepared by the method of House et al. (1972).

Adsorption assays were initiated by incubating 100 μl of an appropriate dilution of membranes with 100 μl of either hybridoma supernatant or a 1:150 dilution of ascites fluid at 4°C overnight. Dilutions were in phosphate-buffered saline (PBS) + 0.02% azide. After removing the membranes by centrifugation in an Eppendorf microfuge for 2 min at 4°C, 30-μl aliquots of the supernatant were assayed for the presence of specific antibody in a Sepharose 6B RIA (Moore et al., 1982). The Sepharose 6B assay is a solid phase RIA in which microtiter wells are coated with the Sepharose 6B fraction from the sodium channel purification procedure and subsequently reacted with mAb, rabbit anti-mouse IgG, and ^{125}I-protein A.

Binding of rabbit anti-p250 antiserum to membranes was measured in a solid phase assay. Thirty microliters of membranes diluted in 25 mM NaCl, 1 mM EDTA, 30 mM Tris, pH 8, were used to coat microtiter wells overnight at 4°C. Wells were washed with PBS + 0.05% Tween 20 and incubated for 45 min with PBS containing 0.05% Tween 20, 0.1% bovine serum albumin, and 0.1% Triton X-100. After incubation with either rabbit anti-p250 antiserum or preimmune serum diluted 1:200 in PBS/Tween/BSA/Triton for several hours at room temperature, wells were then washed with PBS/Tween and incubated with 100,000 cpm of ^{125}I-protein A in PBS/Tween plus 0.25% gelatin. Bound radioactivity was removed with 2 N NaOH and measured in a gamma counter.

Immunocytochemistry. Tissue from the caudal aspect of the electroplax (organ of Sachs) was fixed on 0.1 M lysine, 0.01 M sodium metaperiodate containing 0.05% paraformaldehyde (McLean and Nakane, 1974) for 1 hr at 4°C or was used unfixed. Tissue was frozen and 10-μm sections were cut with a cryostat, parallel to the long

axis of the electroplax. Sections were dried onto subbed coverslips and incubated with hybridoma supernatant, followed by affinity-purified rabbit anti-mouse IgG (4 μg/ml) and rhodamine-labeled goat anti-rabbit IgG (N. L. Cappel Laboratories, Cochranville, PA) diluted 1:40. Dilutions were made with PBS + 10% newborn calf serum. Sections were mounted in UV inert mountant and viewed with a Zeiss fluorescence microscope under epi-illumination with a × 63 or × 40 objective.

Results

Specificity of mAb 5F3 and rabbit anti-p250 serum. Monoclonal antibody 5D10 has previously been shown to react with a component of the eel electroplax sodium channel by virtue of its ability to immunoprecipitate the STX-binding component (Moore et al., 1982). Furthermore, it was shown to bind exclusively to a 250,000-dalton component (p250) as identified on SDS-polyacrylamide gels. Using similar assays, mAb 5F3 has also been shown to react specifically with the eel channel. To detect immunoprecipitation of the STX-binding component, solubilized electroplax membranes were saturated with [^{3}H]STX and incubated with increasing amounts of mAb (prepared as a hybridoma supernatant). The antibody was then precipitated by adding an equivalent amount of rabbit anti-mouse IgG antiserum followed by 3.5% polyethylene glycol, and the immune complexes were separated by centrifugation through a glycerol cushion and counted. The results of such an assay using mAb 5F3 are shown in Figure 1. 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 mAb 5F3 added. Nonspecific background immunoprecipitation was determined under identical conditions, using hybridoma supernatants containing monoclonal IgG1 antibodies directed against an unrelated bovine pituitary antigen (Lemke and Brockes, 1981) (Fig. 1). Furthermore, the radioactivity precipitated by 5F3 was not due to the precipitation of unbound [^{3}H]STX because the signal was abolished when the solubilized electroplax membranes were eliminated from the assay mixture (data not shown).

Antibody 5F3 was shown to bind exclusively to the p250 protein by Western blot analysis on electroplax membrane proteins. Proteins from each of the four stages of purification of the eel channel (membranes, detergent extract, DEAE fraction, and Sepharose 6B fraction (Agnew et al., 1978)) were separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979). The paper was sequentially reacted with 5F3 antibody, rabbit anti-mouse IgG and ^{125}I-labeled protein A, and then autoradiographed (Fig. 2). At each stage of the purification, 5F3 antibody reacted with a diffuse band of M_r ~250,000 (the p250 protein), and this band was not stained by an unrelated control IgG1 mAb. None of the other numerous polypeptide components present in the various fractions reacted detectably with 5F3 even in heavily loaded lanes (e.g., Fig. 2, *lane 1*).

A rabbit antiserum against an SDS-gel-purified p250 preparation (Moore et al., 1982) was also tested for specificity by Western blot analysis. Electroplax membrane proteins from a non-ionic detergent extract fraction and the Sepharose 6B fraction were separated on SDS-polyacrylamide gels, transferred to nitrocellulose paper, and reacted with rabbit anti-p250 serum (1:1000 dilution) followed by ^{125}I-protein A. After autoradiography there was specific reaction only with the p250 protein (Fig. 3). Preimmune serum had no binding under these conditions. Immunoprecipitation assays demonstrated the ability of this antiserum to precipitate the STX-binding component (data not shown).

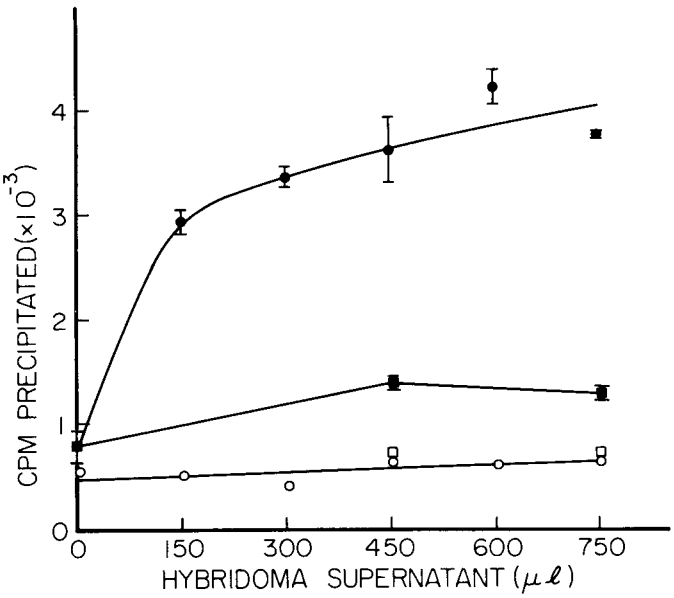

Figure 1. Immunoprecipitation of the STX-binding component by mAb 5F3. A crude detergent extract of electroplax membranes was saturated with [^{3}H]STX in the presence or absence of excess cold TTX (see Moore et al., 1982) and incubated with varying amounts of hybridoma supernatant. Immune complexes were precipitated by addition of rabbit anti-mouse IgG and 3.5% polyethylene glycol and were pelleted by centrifugation through a glycerol cushion. Radioactivity associated with the pellet is plotted against the volume of hybridoma supernatant. ●, mAb 5F3 in the absence of unlabeled TTX; ○, mAb 5F3 in the presence of unlabeled TTX; ■, control IgG1 mAb in the absence of unlabeled TTX; □, same control antibody in the presence of unlabeled TTX. *Error bars* denote the standard deviation about the mean of triplicate points.

These results indicate that mAb 5F3 and the rabbit anti-p250 serum, like mAb 5D10, react specifically with the p250 component of the eel sodium channel.

Cross-reaction studies. Assays were performed to assess the immunological cross-reaction between the eel electroplax sodium channel and sodium channels from other sources. Cross-reaction of mAbs 5D10 and 5F3 were measured with a quantitative adsorption assay. In this assay, a limiting dilution of antibody was incubated with varying amounts of membranes prepared from different tissues and different species. The membranes were pelleted by centrifugation and the supernatant was assayed for residual antibody in a solid phase binding assay (the Sepharose 6B RIA (Moore et al., 1982)). If the membranes display the antigenic determinant recognized by the mAb, then the antibody should be depleted by the adsorption. Results of such an experiment, in which mAb

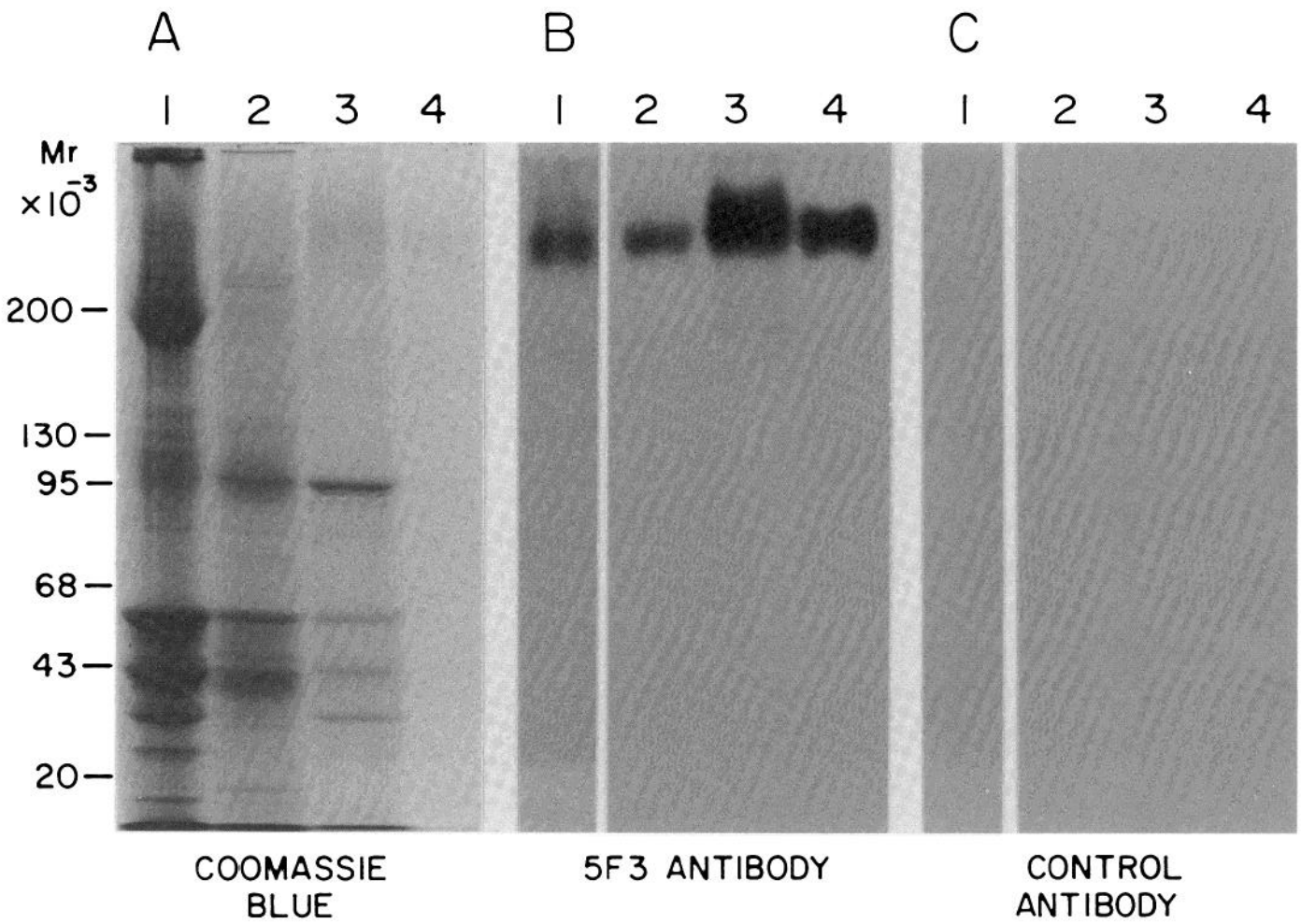

Figure 2. Western blot of electroplax proteins with mAb 5F3. Material from each of the four steps in the sodium channel purification procedure was fractionated in 4 to 12% gradient polyacrylamide gels under non-reducing conditions. Gels were either stained with Coomassie blue or electrophoretically transferred to nitrocellulose paper. The paper was then reacted with hybridoma supernatant followed by rabbit anti-mouse IgG and ^{125}I-protein A. *Lanes 1, 2, 3,* and *4* contain membranes, Lubrol extract, DEAE fraction, and Sepharose 6B fraction, respectively. *A*, Coomassie blue-stained gel. *B*, Autoradiogram of a nitrocellulose transfer from a gel similar to the one in *A*, stained with mAb 5F3. *C*, Autoradiogram of an identical nitrocellulose transfer stained with a control IgG1 mAb. *Lanes B1* and *C1* were from one experiment, *lanes B2* to *B4* and *C2* to *C4* were from an independent experiment.

5D10 was adsorbed with eel electroplax membranes or with crude synaptosomal membranes from rat brain or frog brain, are shown in Figure 4. The data are plotted as cpm bound in a Sepharose 6B RIA performed on the adsorbed supernatant versus the number of TTX-binding sites present in the adsorbing membranes. The results clearly show that, although 5D10 was effectively adsorbed by electroplax membranes, the antibody did not bind detectably to membranes from rat brain or frog brain. Similar assays showed a lack of cross-reaction with chick brain membranes. The *horizontal solid line* in Figure 4 is the value obtained following adsorption with rat kidney membranes. Essentially identical results were obtained when this adsorption assay was performed with mAb 5F3. Two important points can be made from these adsorption experiments. First, they demonstrate that Na^+ channels from eel electroplax and channels from rat brain, frog brain, or chick brain are immunologically distinct. Second, these mAbs recognize the eel Na^+ channel in its native membrane-integrated form. Since the original immunogen was a solubilized preparation, it was possible that the antigenic sites recognized by these mAbs would be inaccessible in the membrane state; the adsorption experiment, however, demonstrates that this is not the case. Similar experiments using the rabbit anti-p250 antiserum demonstrated that greater than 90% of its anti-channel activity could be adsorbed by electroplax membranes.

To investigate the cross-reactivity of the rabbit anti-p250 antiserum, a solid phase RIA was used. Membranes from the source to be tested were coated onto plastic microtiter wells. The coated wells were then reacted with either rabbit anti-p250 serum or preimmune serum from the same rabbit. Wells were then washed and reacted with ^{125}I-protein A. The results (Fig. 5) were plotted as specifically bound radioactivity (immune-preimmune) versus concentration of TTX-binding sites in the membrane preparation used to coat the wells. In the experi-

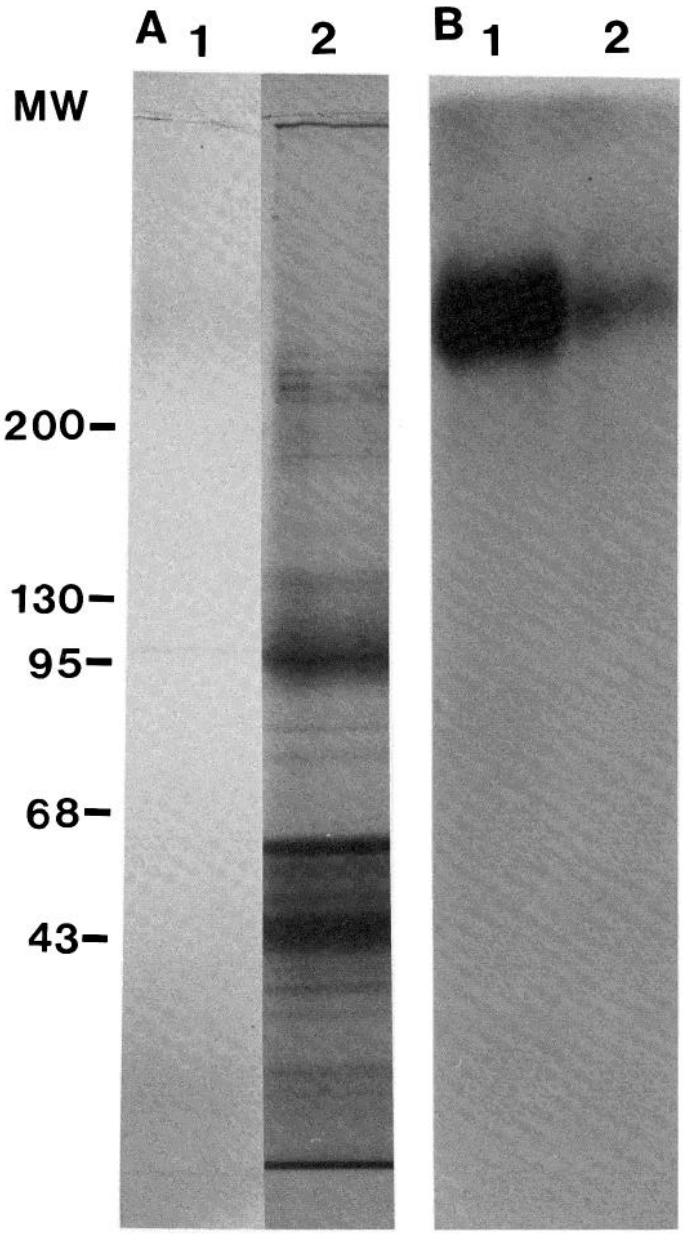

Figure 3. Western blot of electroplax proteins probed with rabbit anti-p250 antiserum. Sepharose 6B fraction (*lane 1*) and Lubrol extract of membrane proteins (*lane 2*) were fractionated on 4 to 12% gradient polyacrylamide gels and either stained with Coomassie blue or electrophoretically transferred to nitrocellulose paper. The paper was then reacted with a 1:1000 dilution of rabbit anti-p250 antiserum and ^{125}I-protein A. *A*, Coomassie blue-stained gel. *B*, Autoradiogram of nitrocellulose transfer.

ment of Figure 5, binding to eel electroplax membranes is compared to that for rat crude synaptosomal membranes. There was detectable binding of the serum to the rat brain membranes, but the displacement between the two curves suggests that this binding was at a level only ~0.1% of that seen with electroplax membranes. No specific binding was seen with rat liver membranes. The rabbit anti-p250 serum also bound to crude brain synaptosomal membranes from chick and frog, but the mag-

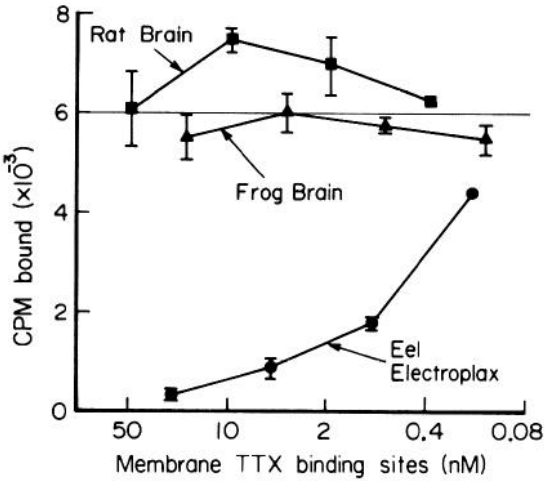

Figure 4. Cross-reaction of mAb 5D10 measured by quantitative adsorption assay. A limiting dilution of 5D10 antibody was incubated with varying amounts of membranes from the indicated sources. After pelleting the membranes, 30 μl of the supernatants were assayed for residual antibody in a Sepharose 6B RIA. Data are plotted as cpm bound in the Sepharose 6B RIA versus the concentration of TTX-binding sites in the adsorption mixture. Adsorptions were performed with: ●, eel electroplax membranes; ■, rat brain membranes; ▲, frog brain membranes. *Error bars* denote the standard deviation about the mean of triplicate points.

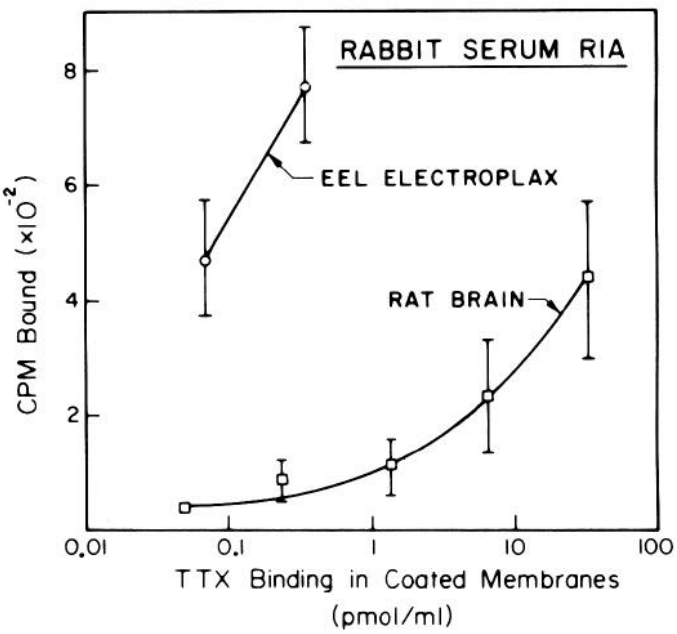

Figure 5. Cross-reaction of rabbit anti-p250 antiserum measured in a solid phase binding assay. Plastic microtiter wells were coated with varying amounts of membrane from either eel electroplax or rat brain. The coated wells were reacted with a 1:200 dilution of rabbit anti-p250 antiserum or preimmune serum followed by ^{125}I-protein A. Data are plotted as specific cpm bound (cpm bound with anti-p250 serum minus cpm bound with preimmune serum) versus the concentration of TTX-binding sites present in the membranes used to coat the wells. ○, wells coated with eel electroplax membranes; □, wells coated with rat brain membranes. *Error bars* denote the standard deviation about the mean of triplicate points.

nitude of this binding was comparable to the low level observed with rat brain. These experiments indicate that even using a polyclonal antiserum, there is only very limited immunological cross-reaction between the Na^+ channel of eel electroplax and the Na^+ channel from the brains of a number of other species.

We also tested the cross-reaction of these monoclonal and polyclonal antibodies with rat skeletal muscle sodium channels in adsorption assays. (The eel electroplax is embryologically derived from myogenic tissue (Keynes, 1957).) No cross-reaction was observed, although these assays had limited sensitivity due to a low specific STX-binding activity in the muscle membrane preparations. These negative results are in accord with our failure to observe immunocytochemical staining of muscle with these antibodies (see "Discussion").

Immunocytochemical studies. mAbs 5D10 and 5F3 and the rabbit anti-p250 serum were all tested for their ability to visualize sodium channels in frozen sections of the eel electroplax; the sections were prepared from the caudal aspect of the electric organ, known as the organ of Sachs (Luft, 1957). The electroplax is a tissue composed of flattened cells (electrocytes) which are organized into stacks running in the rostral-caudal direction. Frozen sections were made parallel to the long axis of the electrocyte stacks and the sections were reacted with either (*1*) monoclonal hybridoma supernatant followed by affinity-purified rabbit anti-mouse IgG (4 μg/ml) and goat anti-rabbit IgG coupled to rhodamine, or (*2*) rabbit serum (anti-p250 or preimmune 1:200 dilution) followed by goat anti-rabbit IgG coupled to rhodamine. The sections could then be examined under phase contrast or rhodamine fluorescence optics.

When staining was performed with mAb 5D10, no rhodamine fluorescence was ever seen using either fixed or unfixed tissue. This result was surprising in light of the adsorption evidence (Fig. 4) that 5D10 recognizes determinants that are accessible when the channel is in its native membrane-integrated state. Sodium channels were successfully visualized by reaction with rabbit anti-p250 serum followed by the rhodamine-coupled second antibody. Clearer images, however, were obtained with mAb 5F3 as primary antibody. (Immunocytochemical staining of eel electrocytes by a rabbit anti-electroplax sodium channel serum has also been reported recently by Ellisman and Levinson (1982).) Figure 6, *left* and

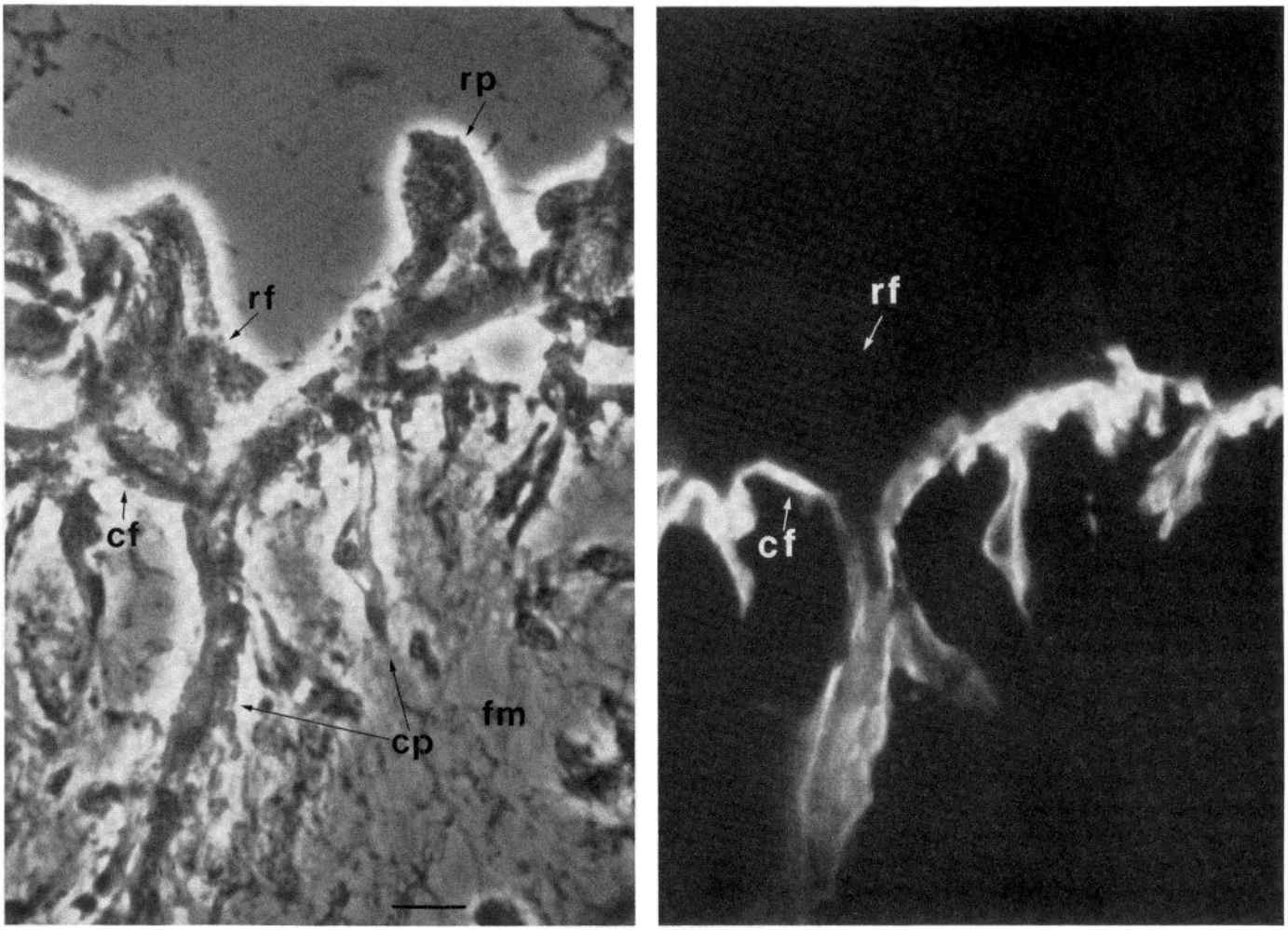

Figure 6. Eel electrocyte stained with mAb 5F3. A frozen section from the organ of Sachs was reacted with 5F3 supernatant, affinity-purified rabbit anti-mouse IgG, and rhodamine-conjugated goat anti-rabbit IgG. *Left*, phase optics; *right*, rhodamine optics. *cf*, caudal face; *rf*, rostral face; *cp*, projection off of the caudal face; *rp*, projection off of the rostral face; *fm*, filamentous material. Immunoreactivity is limited to the caudal face. *Calibration*, 10 μm.

right, consists of photographs, taken with phase contrast and rhodamine optics, respectively, of an electrocyte stained with mAb 5F3 as described above. Sodium channels, indicated by intense fluorescence, are associated solely with the caudal face of the electrocyte. Note also that all of the projections on the caudal face are stained with the antibody. Immunocytochemical experiments using the rabbit anti-p250 serum also showed specific staining exclusively on the caudal face. These results provide an explanation for the physiological results of Keynes and Martins-Ferreira (1953), who showed that action potentials are generated across the caudal but not the rostral face. When similar sections were reacted with IgG1 mouse mAbs directed against unrelated antigens, no fluorescence was observed, demonstrating the specificity of the 5F3 immunoreactivity (Fig. 7).

Although most of the caudal face membrane of these electrocytes is stained by mAb 5F3, there are regions devoid of antibody reaction. In Figure 8 (*left*), the caudal face of the electrocyte has two prominent projections with a region of smooth membrane between them. 5F3 immunoreactivity (Fig. 8, *right*) is strictly localized to the projections, while the membrane between them is negative. Areas of caudal face which are 5F3 negative are generally delimited by abrupt transitions between fluorescent and nonfluorescent regions as is the case in Figure 8, *right*. Occasionally, small patches of fluorescence are seen within the 5F3-negative regions.

One morphological feature which correlates with the presence of 5F3 immunoreactivity is the presence of filamentous material that is extracellular to the electrocyte but which is localized to the caudal side of each cell. This material can be seen in Figures 6 (*left*) and 7 (*left*), and in association with the caudal projections in Figure 8 (*left*). Note the paucity of this material in association with the 5F3-negative membrane in Figure 8.

Discussion

We have investigated the binding properties of two monoclonal antibodies and one rabbit antiserum, each of which recognizes the eel electroplax sodium channel. The specificity of these immunoreagents has been assessed in two ways. First, they were shown to precipitate the STX-binding component from detergent extracts of electroplax membranes. This demonstrates that these reagents do in fact recognize the eel sodium channel. Second, their reactivity was investigated by Western blot analysis. When electroplax membrane proteins were separated by SDS-gel electrophoresis and reacted with these antibodies, detectable reaction was seen only with the p250 band (Figs. 2 and 3).

Quantitative adsorption assays indicated that whereas mAbs 5D10 and 5F3 bind to the electroplax sodium channel, they have no detectable affinity for rat brain, frog brain, or chick brain channels. Binding assays in which plastic wells coated with excitable membranes from various sources were reacted with rabbit anti-p250 serum demonstrate only a very slight cross-reaction (~0.1%) between the eel channel and channel from rat brain, frog brain, or chick brain. The value of 0.1% cross-reaction must be considered an upper bound, since in this assay, information concerning binding specificity has been sacrificed in order to gain greater sensitivity. In the far less sensitive precipitation assay where reaction with the STX-binding component is directly monitored (i.e., assay of Fig. 1), no precipitation of rat brain channel by anti-p250 serum can be detected. Similarly, Western blots of membrane proteins from rat, frog, or

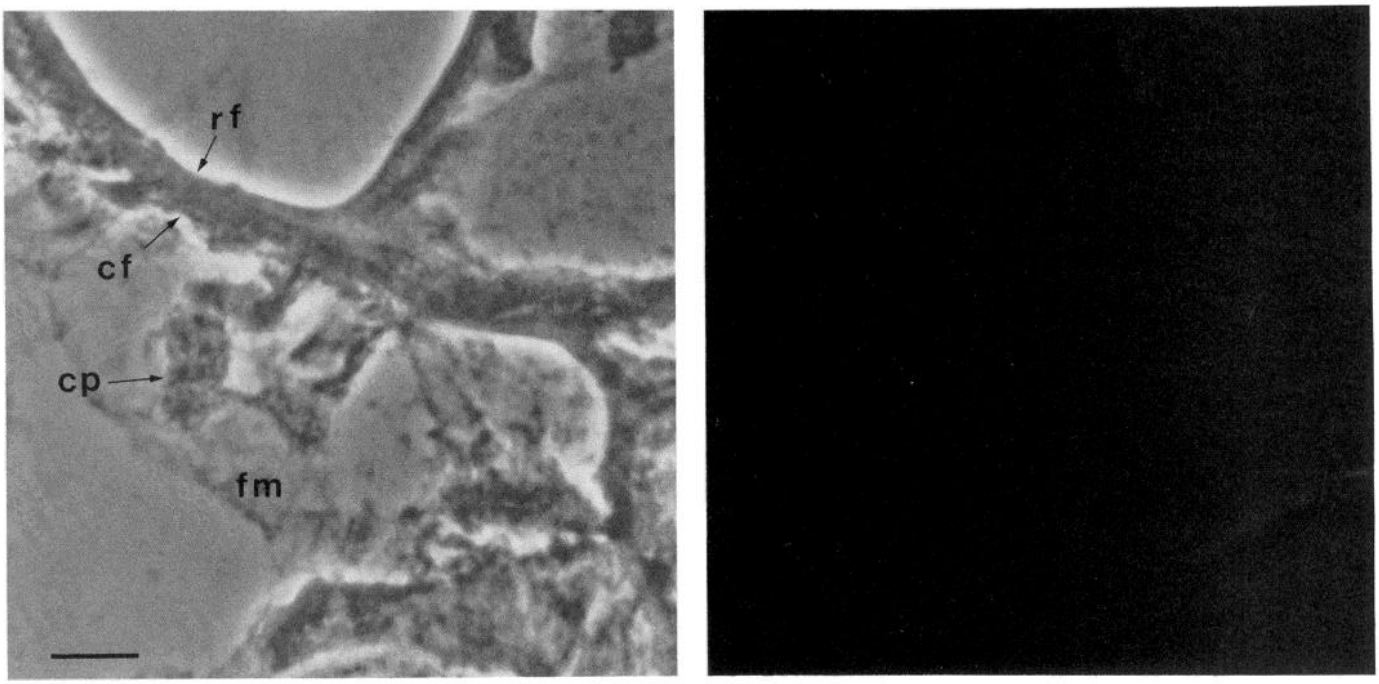

Figure 7. Frozen section of an eel electrocyte stained with a control mouse IgG1 mAb, affinity-purified rabbit anti-mouse IgG, and rhodamine-coupled goat anti-rabbit IgG. *Left*, phase optics; *right*, rhodamine optics. Abbreviations are as in Figure 6. *Calibration*, 10 μm. No immunofluorescence is observed.

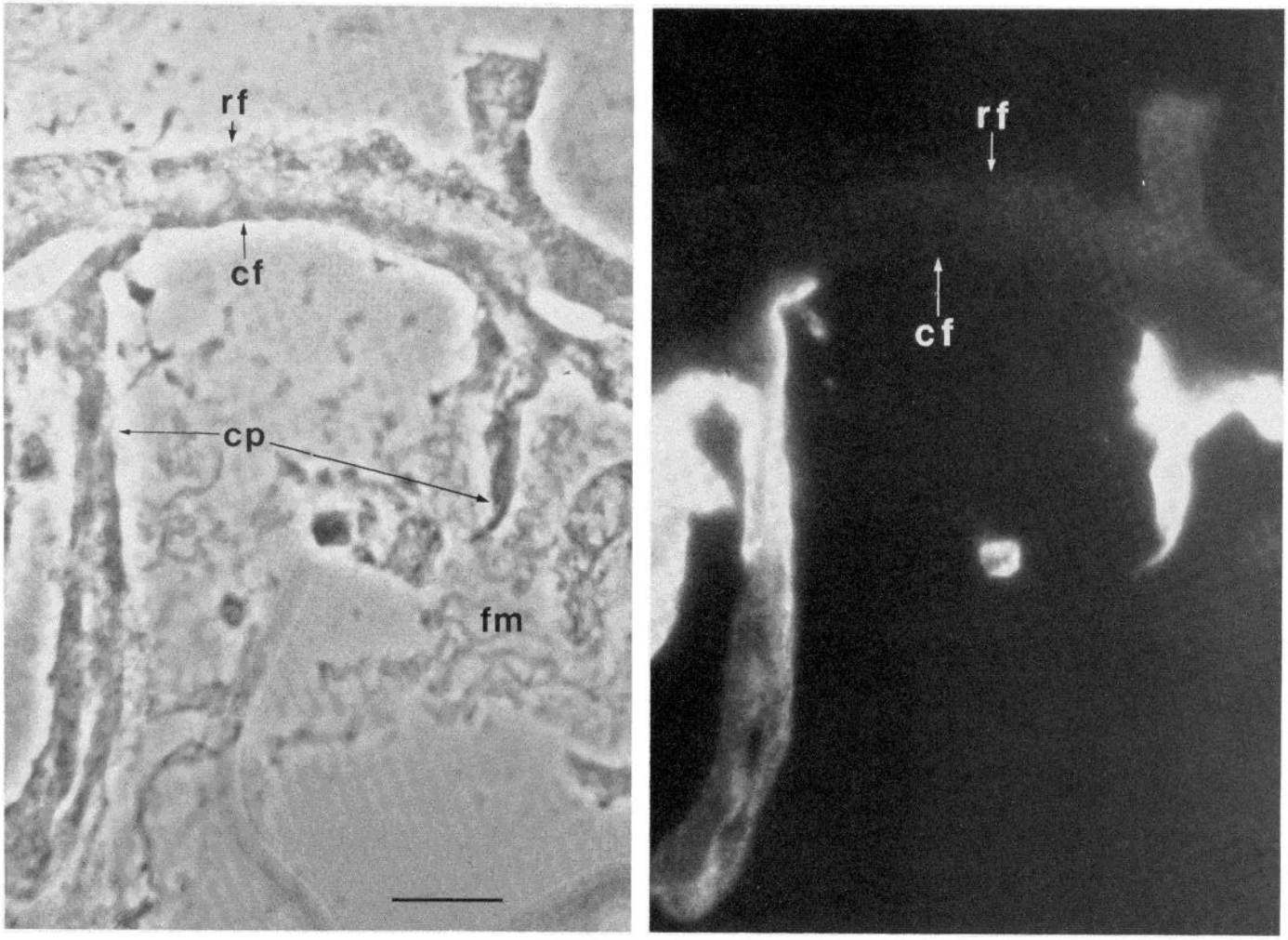

Figure 8. Frozen section of an eel electrocyte stained with mAb 5F3 affinity-purified rabbit anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG. *Left*, phase optics; *right*, rhodamine optics. Abbreviations are as in Figure 6. Immunoreactivity is limited to the caudal projections and is absent from the membrane between them. *Calibration*, 10 μm.

chick brain show no clear reaction when probed with anti-p250 antiserum or with 5D10 or 5F3 (unpublished results). These experiments show that eel electroplax sodium channels are immunologically distinct from brain sodium channels in all species so far tested.

The very limited cross-reaction observed in radiolabeled binding assays is in accord with our failure to visualize cytochemically any binding of these reagents to a variety of excitable tissues. Indeed, we have been able to visualize binding of anti-electroplax sodium channel antibodies only in the electroplax itself (Figs. 6 and 8). Using either fluorescent anti-immunoglobulins or the peroxidase-antiperoxidase technique (Sternberger, 1979), we have been unable to visualize binding of any of our three reagents to cultured rat or chick neurons, cultured rat or chick myotubes, or frozen sections of rat skeletal muscle, myelinated nerve from rat or frog, or rat hippocampus. Perhaps surprisingly, no reaction could be detected between mAb 5F3 and sections of eel spinal cord, eel muscle, or nerves which innervate the electroplax. Intense 5F3 immunoreactivity was easily detected on the caudal face of electrocytes, however (Figs. 6 and 8). Negative results were also obtained with anti-p250 antiserum staining of eel spinal cord and electroplax nerve; there was faint staining of eel muscle. These results are in contrast to those of Ellisman and Levinson (1982), who did see reaction between their anti-electroplax sodium channel antiserum and the node of Ranvier in eel spinal cord axons. Their antiserum was generated against a native channel preparation, whereas our anti-p250 serum was generated against SDS-denatured material. Perhaps antibodies to the native channel have a wider binding repertoire than do those generated against the denatured channel. The antiserum used by Ellisman and Levinson (1982), however, was generated against material that was less pure than the SDS-gel-purified immunogen used to make the anti-p250 serum, and it is difficult to assess the absolute specificity of their serum in the absence of a Western blot analysis against total electroplax membrane proteins.

The very limited cross-reaction seen in these experiments contrasts sharply with immunological results obtained with the acetylcholine receptor. In that molecule, there exists a highly conserved yet highly immunogenic region which accounts for significant cross-reaction between acetylcholine receptors of eel electroplax and

mammalian muscle (Tzartos et al., 1981). To date, the anti-p250 antiserum and our limited sample of mAbs provide no evidence for such a region in the sodium channel.

Quantitative adsorption experiments with 5D10 (Fig. 4) and with mAb 5F3 and rabbit anti-p250 antiserum demonstrate that all three reagents bind to channel determinants that are exposed while the channel is embedded in the membrane. It was possible that, since the immunizations were performed with solubilized channel, the antibodies might react primarily with determinants normally hidden in the plane of the bilayer. Since the adsorptions were performed in the absence of detergents, the antibody-antigen reactions observed must have involved exposed determinants. We cannot say, however, whether a given determinant is on the cytoplasmic or extracellular side of the membrane.

The distribution of sodium channels in frozen sections of the electroplax (organ of Sachs) could be visualized with rabbit anti-p250 serum or with mAb 5F3 but not with mAb 5D10; the sharpest images with lowest background staining were obtained with mAb 5F3. Presumably, some aspect of the frozen sectioning procedure destroyed the determinant recognized by mAb 5D10, since from the adsorption experiments, 5D10 clearly bound to an exposed determinant. The cytochemical results indicate that sodium channels are exclusively localized to the caudal face of each electrocyte as suggested by electrophysiological experiments which showed action potential generation only across the caudal face (Keynes and Martins-Ferreira, 1953). Recently, Ellisman and Levinson (1982) also described the immunochemical localization of sodium channels to that face. All of the projections off of the caudal face were brightly stained by mAb 5F3 and, indeed, most of each caudal face was 5F3 positive. Of particular interest, however, are the occasional regions of caudal face which lack sodium channel immunoreactivity. These regions also seem to lack the extracellular filamentous material that is in association with most of the caudal face membrane. This material is also absent from the entire rostral face. Within this material are the axons which innervate the electrocytes (Luft, 1957).

An intriguing possibility is that this extracellular material interacts with the electrocyte in a process which leads to localization of sodium channels. Interactions between extracellular matrix components and myotubes have been implicated in the clustering of acetylcholine receptors at neuromuscular junctions (Burden et al., 1979; Rubin and McMahan, 1982).

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