

Evidence for a Polarity in the Distribution of Proteins from the Cytoskeleton in *Torpedo marmorata* Electrocytes

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Abstract. The subcellular distribution of the 43,000-D protein (43 kD or ν_1) and of some major cytoskeletal proteins was investigated in *Torpedo marmorata* electrocytes by immunocytochemical methods (immunofluorescence and immunogold at the electron microscope level) on frozen-fixed sections and homogenates of electric tissue. A monoclonal antibody directed against the 43-kD protein (Nghiễm, H. O., J. Cartaud, C. Dubreuil, C. Kordeli, G. Buttin, and J. P. Changeux, 1983, *Proc. Natl. Acad. Sci. USA*, 80:6403–6407), selectively labeled the postsynaptic membrane on its cytoplasmic face. Staining by anti-actin and anti-desmin antibodies appeared evenly distributed within the cytoplasm: anti-desmin antibodies being associated with the network of intermediate-sized filaments that spans the electrocyte, and anti-actin antibodies making scattered clusters throughout the cytoplasm without preferential labeling of the postsynaptic

membrane. On the other hand, a dense coating by anti-actin antibodies became apparent on the postsynaptic membrane in homogenates of electric tissue pointing to the possible artifactual redistribution of a soluble cytoplasmic actin pool.

Anti-fodrin and anti-ankyrin antibodies selectively labeled the non-innervated membrane of the cell. F actin was also detected in this membrane. Filamin and vinculin, two actin-binding proteins recently localized at the rat neuromuscular junction (Bloch, R. J., and Z. W. Hall, 1983, *J. Cell Biol.*, 97:217–223), were detected in the electrocyte by the immunoblot technique but not by immunocytochemistry.

The data are interpreted in terms of the functional polarity of the electrocyte and of the selective interaction of the cytoskeleton with the innervated and non-innervated domains of the plasma membrane.

THE postsynaptic membrane of the neuromuscular junction and of the electromotor synapse corresponds to a local differentiation of the plasma membrane characterized by an accumulation of the nicotinic acetylcholine receptor (Ach-R).¹ This membrane specialization persists for a long period after denervation (reviewed in references 10 and 18), indicating that physical constraints maintain the Ach-R molecules in place and, in particular, prevent against their lateral diffusion. Interactions of the Ach-R with extrinsic components from the extracellular matrix (13, 55) and/or with the cytoskeleton (17, 48, 67) have been postulated to contribute to this differentiation process.

Fish electric organs have provided a uniquely rich source of cholinergic synapses that already led to the characterization of several of their molecular components and, thus, constitute an adequate system to analyze their interactions. The adult electric tissue (21) from *T. marmorata* is made up of multi-nucleated units or electrocytes that receive nerve endings exclusively on their ventral surface, while the other side of the

cell—the non-innervated face—is specialized in the regeneration of the electrochemical gradient (40). The Ach-R is present exclusively on the innervated membrane, and Na⁺ K⁺ ATPase accumulates on the non-innervated one. These membranes thus constitute fully differentiated and stable domains of the plasma membrane. From this standpoint the disk-shaped electrocyte shows a striking functional and structural polarity (18, 21).

Postsynaptic membrane fractions purified from *Torpedo* comprise essentially the intrinsic Ach-R polypeptides plus few extrinsic components of apparent molecular mass 43 kD (63, 64) named ν proteins (31), which include a strongly membrane-bound cysteine-rich protein named ν_1 (63) and two loosely bound ones: creatine phosphokinase (or ν_2 , 2, 26, 29) and cytoplasmic actin (or ν_3 , 29, 46).² Alkaline extraction of the nonreceptor, peripheral proteins of the membrane causes important structural alterations, such as an increase in rotational (3, 39, 52, 53) and translational (1, 16, 52) mobility and an enhanced sensitivity to heat denaturation (54) and proteolysis (35) of the Ach-R. In addition, after this treatment,

² In the text to follow, 43 kD refers to the membrane-bound ν_1 component.

¹ Abbreviations used in this paper: Ach-R, nicotinic acetylcholine receptor; m Ab, monoclonal antibody.

the cytoplasmic densities which, in situ, appear co-extensive with the Ach-R-rich areas of the membrane, are no longer visible (16, 58). These results suggest that the 43-kD protein represents a major component of the submembrane condensations and may contribute to the immobilization and stabilization of the Ach-R. Biochemical (65, 71) and immunocytochemical (45, 61) data indeed show that the 43-kD protein is located on the cytoplasmic side of the Ach-R-rich domains of the postsynaptic membrane. Cross-linking experiments further suggest a proximity between the subunits of the Ach-R and the 43-kD protein (14). Finally, a protein immunologically related to *Torpedo* 43 kD is also present at mammalian neuromuscular junctions (23, 24).

In the postsynaptic domain of the neuromuscular junction, immunofluorescence experiments revealed that several cytoskeletal proteins including cytoplasmic actin (30), actin-binding proteins, such as α -actinin, filamin, and vinculin (7), talin (60), and a 51,000-D intermediate filament-related protein (12) are also present, thus making plausible an interaction of the postsynaptic Ach-R with the cytoskeleton.

As a first approach to unravel such interactions, we have attempted to identify and localize these components by immunocytochemical methods at the electron microscope level in *T. marmorata* electrocyte. We report that the cortical cytoskeleton is totally asymmetric with respect to the cell surface, the 43-kD protein being exclusively localized at the Ach-R-rich, innervated side, while fodrin, ankyrin, and F actin are observed only at the ATPase-rich, non-innervated side of the cell. These data are interpreted in terms of molecular interactions between membrane proteins from both faces of the cell with components of the cytoskeleton.

Preliminary data on the localization of the 43-kD protein have been published (17, 45).

Material and Methods

Membrane-Cytoskeleton Enriched Fractions

Membrane fractions comprising cytoskeletal elements derived from both innervated and non-innervated faces of the electrocytes were prepared according to Heuser and Salpeter (32) with modifications. Pieces of electric tissue were gently homogenized in a loose glass Potter homogenizer rotating at 800 rpm in 100 mM KCl/5 mM MgCl₂/3 mM EGTA/1 mM EDTA/20 mM Tris-HCl pH 7.4 with anti-proteases 200 μ M phenylmethylsulfonyl fluoride, and 10 U aprotinin, 1 μ g pepstatin A, 10 μ g leupeptin, and 10 μ g antipain per milliliter. Fragments were washed three times in the homogenization buffer, pelleted at 1,000 g for 5 min, and verified at the electron microscope as containing membrane sheets derived from both faces of the electrocytes, and attached cytoskeleton. These fragments were used for immunoblotting and immunogold-labeling experiments.

Antibodies

Human erythrocyte ankyrin was prepared according to Tyler et al. (69). Pig brain spectrin (fodrin) was isolated according to Glenney et al. (27). The antigens were purified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) and injected to rabbits. Antibodies were affinity-purified (Regnoui, F., E. Nguyen, R. Cassoly, and L. A. Pradel, manuscript in preparation). Anti-desmin and anti-actin are mouse monoclonal antibodies (m Ab) directed against chicken gizzard desmin and muscle actin (Grimaud, J. A., and D. Paulin, manuscript in preparation). Anti-filamin and anti-vinculin antibodies were gifts of D. Louvard, Institut Pasteur, Paris (62) and Dr. B. Geiger, Rehovot, Israel (25). Anti-43-kD m Ab had been characterized in a previous report (45). Anti-vimentin and anti-intermediate filaments IgM antibodies derived from human myeloma (20).

Immunoblot

Proteins from the electrocyte fractions were separated by SDS PAGE (0.1% sodium dodecyl sulfate on 8% acrylamide/0.05% bisacrylamide) (36) and

electrophoretically transferred to nitrocellulose paper (pore size 0.45 μ m) in Tris-glycine 20 mM –150 mM, pH 8.6/20% methanol (68). Reversible staining with Ponceau red (19) was used to ensure that an efficient transfer has taken place. The transferred nitrocellulose sheet was saturated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4; PBS) and incubated in miniature containers (Nghiem, H. O., manuscript in preparation) with the antibodies tested (Ig concentration 1–20 μ g/ml). Horseradish peroxidase-conjugated sheep or goat anti-species IgG (H+L) were used as the second antibodies (dilution 1:300–1:500) with 0.5% BSA/7.5% horse serum in PBS/0.1% Tween 20. The blots were thoroughly washed with PBS/0.1% Tween 20, and the color reaction was developed with 3,3'-diaminobenzidine (500 μ g/ml)/0.03% H₂O₂/100 mM Tris-HCl pH 7.6.

Immunofluorescence of Frozen Sections

Freshly dissected columns of electrocytes from adult *Torpedo* electric organ were prefixed with 4% buffered paraformaldehyde for 2 h at 4°C, impregnated with buffered solutions of sucrose 5%, 10%, 20% wt/vol, and rapidly frozen in Freon R22.

Frozen sections (6–8 μ m) were cut in a cryostat (SLEE, London), picked up onto ovalbumin-coated glass slides, dried at room temperature, and stored at –70°C until used. Unfixed tissue was processed in the same way.

Antigens in the frozen sections were detected by indirect immunofluorescence. The sections were preincubated with PBS pH 7.6 containing 10% decplemented goat serum, 1% BSA, and 0.1% Tween 20 for 15 min at room temperature in order to reduce background staining, then incubated with the tested antibodies diluted (final Ig concentration 10–50 μ g/ml) in PBS-1% goat serum –1% BSA –0.1% Tween (incubation buffer), and then rinsed with PBS-Tween (3 \times 5 min). Antibodies were revealed by fluorescein-conjugated goat anti-rabbit IgG (fGAR; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) 1:500 in the incubation buffer.

Tetramethylrhodamine-labeled α -bungarotoxin was routinely used to label Ach-R at the postsynaptic membrane of the electrocyte (49).

When m Ab were used, an intermediate incubation with rabbit anti-mouse Ig (H + L) (50 μ g/ml) were carried out. In order to demonstrate specificity of the antibodies in our tissue, inhibition experiments were performed with antigen-adsorbed antibodies (molar ratio Ab/Ag ~ 1:10). Nonspecific binding of the fluorescein-conjugated IgG was tested with substitution of the specific antibodies by either PBS, non-immune, preimmune sera, or control ascitic fluid.

Sections were rinsed with PBS (3 \times 5 min), mounted in PBS (30% vol/vol) glycerol, and viewed in a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epifluorescent illuminator and filters allowing rhodamine and fluorescein fluorescence to be viewed separately. Original photographs were made using Kodak Ektachrome 400–1600 color films.

Phalloidin Labeling of F Actin

Rhodamine-conjugated phalloidin (Molecular Probes, Inc., 0.1 μ M in PBS) was used to localize F actin in frozen sections of electric tissue, according to Wieland et al. (72).

Immunogold Labeling

Immunogold labeling was performed either with freshly prepared membrane-cytoskeleton fractions (see above), or on cryostat sections. Phenylmethylsulfonyl fluoride was added in buffers throughout the labeling procedure. To reduce the background staining, membranes were preincubated with Tris-HCl-buffered saline (20 mM Tris-HCl, 0.15 M NaCl, pH 7.2; TBS) containing 10% decplemented goat serum and 1% BSA for 15 min, then incubated with the tested antibodies diluted (final Ig concentration 10–50 μ g/ml) in TBS-1% goat serum-1% BSA for 1 h at room temperature and then washed with TBS (3 \times 5 min). Antibody reaction was revealed by incubation with colloidal gold-labeled goat anti-rabbit IgG (GAR G5 or GAR G10, Janssen Pharmaceutica, Beerse, Belgium) (1:20 to 1:40) in the incubation buffer for 1 h at room temperature. Care was taken to remove the possible gold aggregates by centrifugation, according to the manufacturer's instructions. Nonspecific binding of the gold-conjugated IgG was tested by substituting the specific antibodies by either TBS, nonimmune or preimmune sera, control ascitic fluid, or by omitting the intermediate serum when monoclonal antibodies were used.

After immunogold processing, tissue fragments were fixed with 1% buffered glutaraldehyde for 30 min, postfixed with 1% OsO₄ for 45 min at 4°C, and embedded in Epon Araldite. Thin sections (grey to gold) were obtained with an LKB ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD) equipped with a diamond knife, and were carbon coated. Some sections were stained according to Reynolds (50). Alternatively, labeled fragments were prepared for negative staining (1% uranyl acetate).

The immunogold labeling was also carried out with cryostat sections of fixed

tissue, as described by Nakane (43). Sections prepared as for the immunofluorescence experiments were incubated first with TBS pH 8 containing 0.1% BSA and 5% goat serum for 15 min, then with specific antibodies diluted (final Ig concentration 10–50 $\mu\text{g}/\text{ml}$) in the same buffer (but 1% goat serum) for 1 h at room temperature. Antibody reaction was revealed by incubation with GARG 10 (1:10) for 2 h at room temperature. Labeled cryostat sections were washed in PBS, then in 0.2 M phosphate buffer pH 7.4, fixed with 1% glutaraldehyde/0.2% tannic acid, and postfixed with 0.5% OsO_4 for 10 min at 0°C, to preserve actin from the destructive effects of the osmium tetroxide according to Maupin-Szamiar and Pollard (42). Cryostat sections were rapidly dehydrated with ethanol, embedded in Epon-Araldite, and cut with an LKB ultramicrotome into thin sections, which will be observed without any further staining.

Observations were made at 60 or 80 kV with Philips 300 or 400 electron microscopes (Philips Electronic Instruments, Inc., Mahwah, NJ). Pictures were taken on Kodak electron microscope films 4489.

Results

Specificity of the Antibodies Used for Immunocytochemical Localization of Cytoskeletal Proteins in *T. marmorata* Electric Tissue

The antibodies used to localize cytoskeletal elements in *T. marmorata* electrocytes were obtained against antigens puri-

fied from other sources. Their reactivity toward the same or immunologically related antigens in *T. marmorata* electric tissue was tested by immunoblotting of one-dimensional SDS polyacrylamide gels (see Materials and Methods). Fig. 1a shows that for most of the antibodies tested, one mainly stained band was revealed around the expected apparent molecular weight regions: 43,000 D for anti-43-kD m Ab, 47,000 for anti-actin, 130,000 for anti-vinculin, 240,000 for anti-fodrin, and 250,000 for anti-filamin. With anti-ankyrin, besides the 200,000 band, another component of higher molecular weight appeared densely stained (Fig. 1a, lane 5). With anti-desmin one or two bands in the 50,000-D region were revealed depending on the preparations tested. (This variation would have resulted from a degradation process occurring during the preparation steps.) The reactivity of the cytoskeletal-enriched fraction with anti-filamin (Fig. 1a, lane 8) was rather weak. No reactivity with a human monoclonal anti-vimentin antibody was detected. A monoclonal anti-intermediate filament antibody also reacted with the cytoskeletal enriched fractions (Fig. 1a, lane 1).

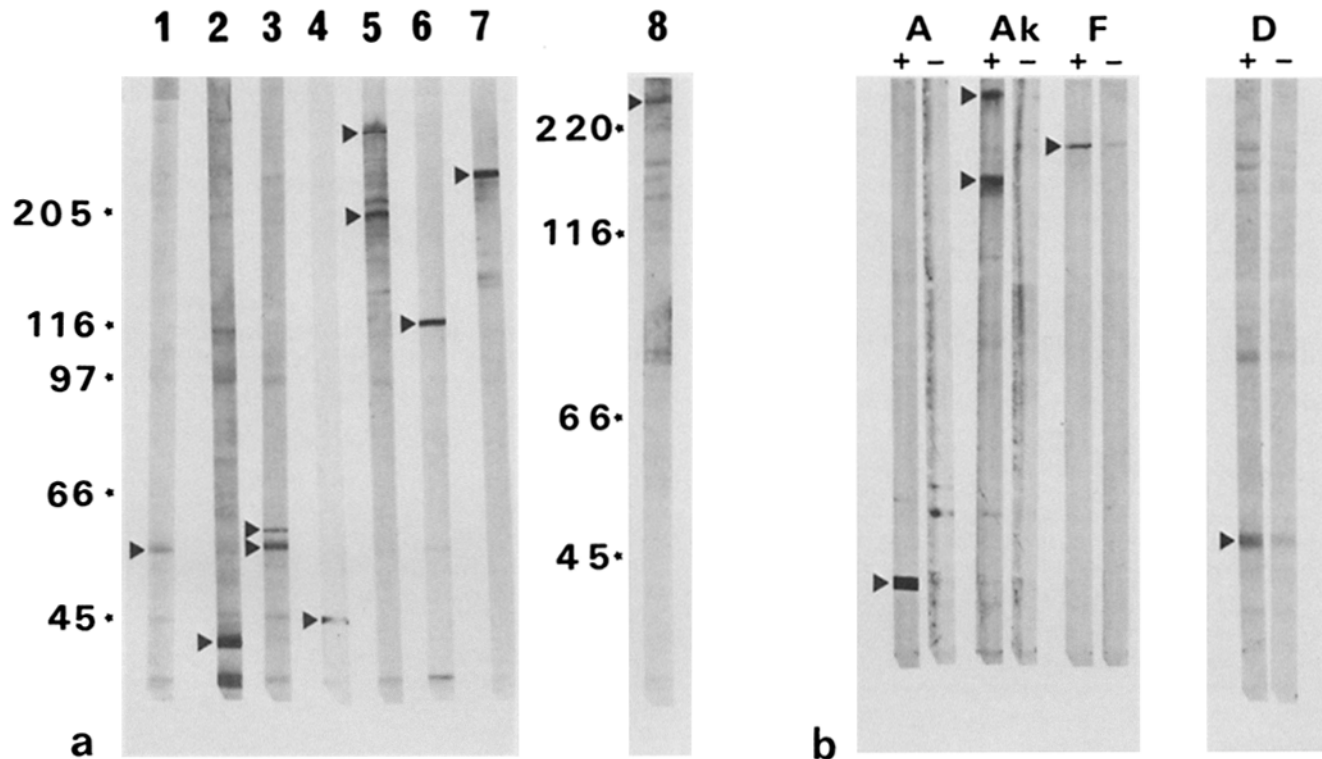


Figure 1. (a) Immunodetection of the 43-kD protein and cytoskeletal polypeptides from *Torpedo marmorata* electrocyte. Polypeptides of membrane fractions from *Torpedo marmorata* electrocytes containing cytoskeletal elements were separated on a SDS PAGE, electrotransferred to nitrocellulose paper, and incubated with the tested antibodies (1–20 $\mu\text{g}/\text{ml}$) (immunoblot). The horseradish peroxidase staining was used to detect the reactive polypeptide bands after incubation with anti-intermediate filaments (lane 1), anti-43-kD (lane 2), anti-desmin (culture supernate, lane 3), anti-actin (lane 4), anti-ankyrin (lane 5), anti-vinculin (lane 6), anti-fodrin (lane 7), and anti-filamin (lane 8) antibodies. Stars indicate the migration distances of molecular weight markers. The second strongly labeled band detected in lane 2 may correspond to degradation products (see reference 45). Note that for each antibody tested, the reactive polypeptides (arrowheads) were detected at the expected apparent molecular weight regions. Two reactive polypeptide bands were always detected with anti-ankyrin antibodies. Anti-desmin m Ab reveals one or two polypeptide bands, depending on the membrane preparation. (b) Inhibition tests for the demonstration of specificity of the immunodetection of polypeptide bands from *Torpedo* electrocyte fractions with anti-cytoskeleton antibodies. Antibodies were preincubated with an excess of the purified related antigens (molar ratio Ab/Ag = 1/10) then tested by immunoblotting with replicas of electrocyte fractions (lane -). Control reactions (lanes +) were performed with the same antibodies on replicas of electrocyte fractions, but without preincubation with the related antigens. Lanes A stand for anti-actin, lanes Ak for anti-ankyrin, lanes F for anti-fodrin, and lanes D for anti-desmin antibodies. Note that a strong or complete inhibition is observed in all lanes (-), indicating the specificity of the immunoreaction observed with the four antibodies.

The specificity of the anti-43-kD m Ab was characterized on two-dimensional immunoblots (see reference 45). The specificity of the immune reaction of the other antibodies was verified by an inhibition test. Antibodies were preincubated with the corresponding purified antigens (molar ratio Ab/Ag 1:10) then reacted with replicas of SDS gels from the electrocyte preparations. Extinction of the reactivity of the antibodies with a particular protein band indicated the specificity of the immunoreaction. Fig. 1*b* shows that a complete inhibition of the reaction was observed with anti-actin and anti-ankyrin antibodies. Strong inhibitions were observed with anti-desmin and anti-fodrin. These results confirm the specificity of the staining obtained with the relevant antibodies (cf. Fig. 2).

Localization of Components of the Cytoskeleton in T. marmorata Electrocyte by Indirect Immunofluorescence

The subcellular distribution of the 43-kD protein, desmin, actin, vinculin, filamin, fodrin, and ankyrin was investigated by indirect immunofluorescence on cryostat sections of fixed tissue. Simultaneous localization of the cellular antigens and Ach-R was achieved by double labeling experiments using rhodamine- α -bungarotoxin as a specific marker for the nicotinic receptor and a fluorescein-conjugated second antibody for the studied antigens. Electric tissue was sectioned transversally to facilitate the penetration of the immunoreagents. Such sections further display a regular disposition of innervated and non-innervated membranes which become easy to identify.

In these sections, Ach-R (Fig. 2*a*, left column) co-distributed with the 43-kD protein (middle column) at the ventral surface of the cells (see also reference 23). Desmin-like protein and actin appeared in the cytoplasm (Fig. 2, *b* and *c*); on the other hand, anti-ankyrin and anti-fodrin antibodies reacted preferentially with the non-innervated faces of the cells (Fig. 2, *d* and *e*); the faint labeling of the innervated faces was due to the presence of both antigens in the presynaptic structures (see electron microscope pictures, Figs. 8 and 9). A faint cytoplasmic labeling was also detected with the anti-ankyrin antibody.

Labeling was almost totally abolished after preincubation of the antibodies with the purified antigens (Fig. 2, *b-e*, right column). The two actin-binding proteins tested, vinculin and filamin, were not revealed by immunofluorescence (data not shown). Experiments carried out with unfixed tissue sections yielded similar negative results despite the fact that, as shown in Fig. 1, vinculin and filamin were detected in the homogenates with the same antibodies.

Subcellular Localization of F Actin

Rhodamine-phalloidin was used as a specific marker for F actin in fixed cryostat sections of electric tissue. Fig. 3 shows that most of the fluorescence was observed on the non-innervated membrane. A faint labeling of the innervated face of the cell was also observed. It is probably associated with the nerve endings as for ankyrin and fodrin (Kordeli, E., J. Cartaud, H. O. Nghiêm, and J. P. Changeux, manuscript in preparation). No actin filaments were detected within the cytoplasm. The striking discrepancy between the antibody and the phalloidin labeling of actin suggests that the m Ab only recognized a nonfilamentous form of actin in the electrocyte.

Immunocytochemical Localization at the Electron Microscope Level

The detailed spatial relationships between the plasma membrane and cytoskeletal components was investigated at the electron microscope level by the immunogold technique.

Distribution of the 43-kD Protein. The localization of the 43-kD protein was carried out with crude homogenates of electric organ. At low magnification, only the fragments derived from the innervated face (identified, in particular, by the presence of nerve endings, basal lamina and by its smooth surface) were significantly labeled (Fig. 4). High magnification images showed that the labeling was restricted to the cytoplasmic face of the postsynaptic membrane, a result consistent with biochemical data indicating that the 43-kD polypeptide is a peripheral protein (44) associated with the cytoplasmic face of the membrane (65, 71). Staining appeared continuous on most of the postsynaptic surface with interruptions occurring at the bottom of the folds, when the sections were properly oriented (Fig. 4*b*). The 43-kD polypeptide thus displays a tangential arrangement similar to that of the Ach-R and of the cytoplasmic condensations (15, 57, 59). Observation of negatively stained labeled membrane sheets revealed the presence of numerous cytoskeletal filaments associated with the cytoplasmic surface of the postsynaptic membrane (Fig. 5). In these pictures, labeling was obviously restricted to the membrane domain, the filaments observed being never labeled on their length. We have noted that in areas of high density of labeling, such as that shown in Fig. 5*a*, the gold granules were not always randomly distributed in the membrane plane, but rather arranged into double rows suggestive of the supramolecular organization of the receptor itself (15, 32).

A close look at the pictures disclosed that the bulk of the filaments belongs to the intermediate-sized filaments class. Immunogold study of the cytoskeleton has confirmed that it contains desmin-like filaments (see below). In some areas of the electron microscope grid, we observed a significant number of detached 10-nm filaments that are labeled at their free end with the anti-43-kD m Ab (Fig. 5, *b* and *c*). This end-on labeling is indicative of a physical interaction between the intermediate-sized filaments and the postsynaptic, 43-kD protein-rich membrane coating.

Subcellular Localization of Desmin. In agreement with immunofluorescence pictures, anti-desmin m Ab gave by the immunogold technique intense labeling of the bulk of the intermediate-sized filaments that remained attached to fragments from both innervated and non-innervated membranes on crude homogenates of electric organ. Labeling was restricted to the network of filaments, membranes, as well as other cellular structures being stained at background level (Fig. 6).

Aggregation of the 10-nm filaments was frequently observed after immunostaining. This phenomenon might result from the high antigen concentration, the flexibility of the filaments, and the polyvalency of the antibody. Another consequence of the high concentration of the antigen was that the intermediate filaments were only partially labeled, probably because of the conditions of low Ab/Ag ratio of our experiments. Using the same technique, we were unable to detect vimentin in the same homogenates.

Subcellular Localization of Actin. Immunofluorescence

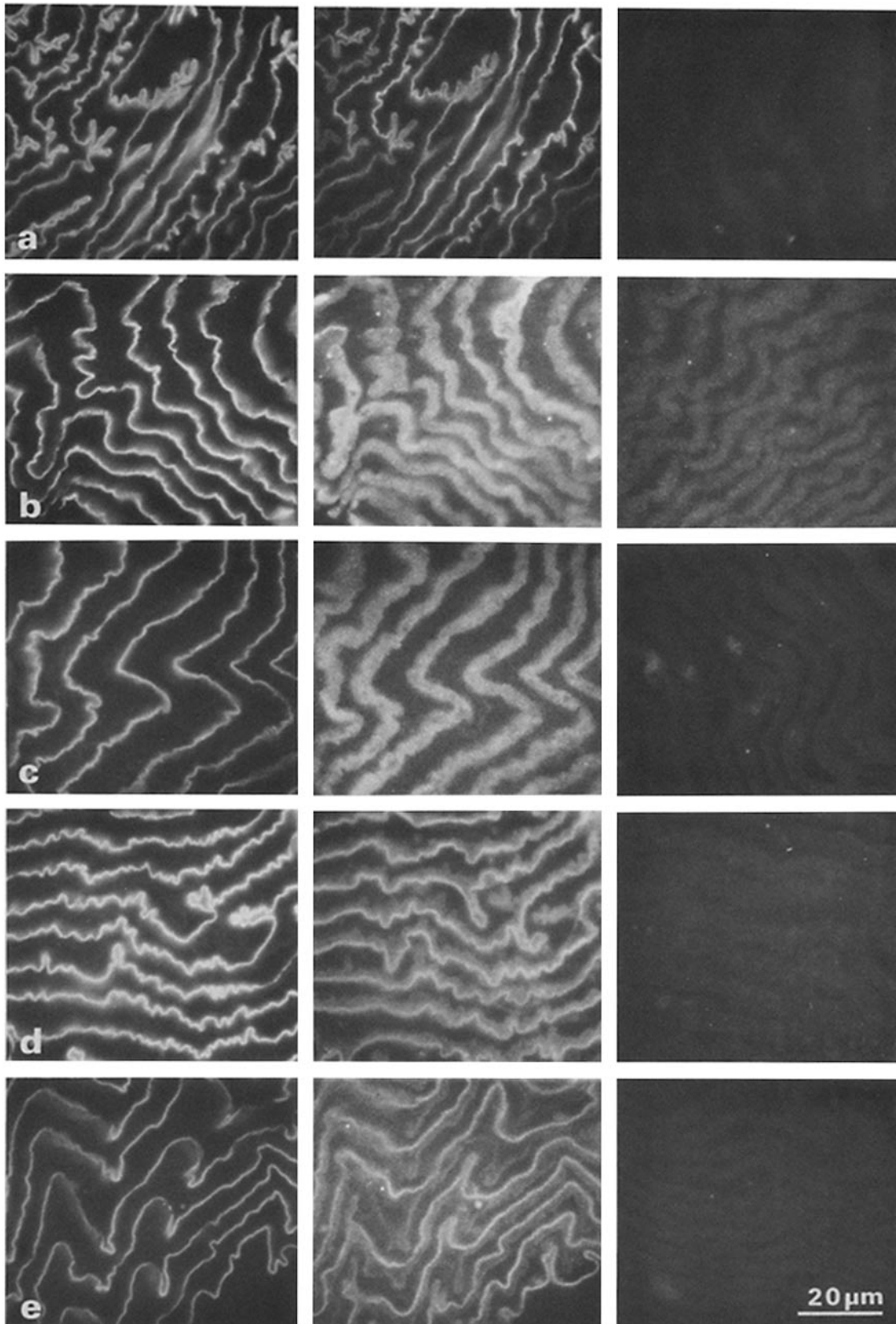


Figure 2. Indirect immunofluorescence localization of the 43-kD protein and cytoskeletal proteins (desmin, actin, ankyrin, and fodrin) in *T. marmorata* electrocytes. Left column, rhodamine-labeled α -bungarotoxin staining; middle column, fluorescein-indirect antibody staining; right column, control. (a) Co-distribution of Ach-R (left column) and 43-kD protein (middle column) on the innervated side of the electrocyte. Control sections (right column) were incubated with non-immune ascitic fluid. Desmin (b) and actin (c) m Abs (middle columns) stain diffusely the whole cytoplasm of the cell. Controls (right columns) were obtained after preincubation of the m Abs with the purified antigens. Anti-ankyrin (d) and anti-fodrin (e) antibodies (middle columns) stain mainly the non-innervated face of the electrocyte. A faint labeling of the innervated face is also observed. The anti-ankyrin antibody also stains the cytoplasm. Controls (right columns) were obtained after preincubation of the antibodies with the purified antigens.

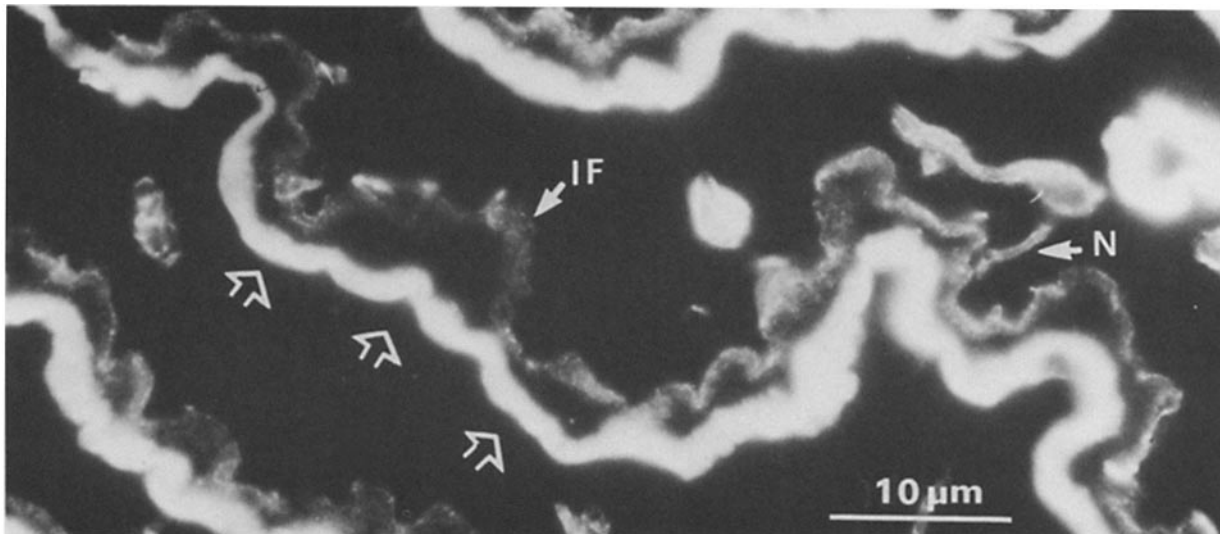


Figure 3. Subcellular localization of F actin by rhodamine-phalloidin fluorescence in cryostat sections. Rhodamine-phalloidin mainly labels the non-innervated membrane of the electrocytes (large arrows). The cytoplasm appears free of actin filaments. A faint and discontinuous labeling at the innervated face (*IF*) probably corresponds to nerve terminals (compare to α -bungarotoxin-labeled postsynaptic membrane in Fig. 2). Labeled ramifications of nerve are also observed (*N*).

pictures achieved with the anti-actin m Ab indicated a cytoplasmic distribution of actin in the electrocytes. Previous biochemical studies showed that the majority of actin in the electrocyte is G actin, which was recovered in the supernatant after tissue fractionation (66). It is thus susceptible to redistribution upon homogenization (see below). To study its cellular distribution at the electron microscope level, homogenization was avoided and the immunological reactions carried out on cryostat sections of fixed tissue (43) (see Materials and Methods). Fig. 7 confirms the cytoplasmic distribution of G actin: staining was never found directly associated with filaments or with cellular membranes. At high magnification, the anti-actin m Ab made small aggregates or rosettes of gold particles dispersed throughout the cytoplasm. No such aggregates were observed in the same series of experiments with antibodies directed against other proteins (compare with Figs. 8 and 9). Thus, this pattern in dispersed clusters most likely reflects the actual distribution of actin in fixed sections. No filaments decorated with the anti-actin m Ab were observed. Such a picture is consistent with the absence of cytoplasmic actin filaments in *Torpedo* electrocytes previously reported by Heuser and Salpeter using a deep-etching replication technique (32).

On the other hand, in tissue homogenates, a discontinuous actin coating became apparent on the cytoplasmic face of the innervated membrane fragments (Fig. 7*d*). The subneural cytoplasm was no longer labeled under these conditions (Fig. 7*e*). Such figures most likely result from a redistribution of the actin pool during the homogenization of the electric tissue.

Subcellular Localization of Ankyrin and Fodrin. Immunogold localization of fodrin and ankyrin was achieved on cryostat sections, following the method described for actin, since as demonstrated by SDS PAGE, these two proteins are partially proteolyzed after homogenization of the electric tissue (data not shown). Figs. 8 and 9 show that with both antibodies, the labeling was primarily associated with the membrane folds at the non-innervated side of the electrocyte. The postsynaptic membrane was never labeled. Accumulation

of gold particles was often noticed within the nerve endings, at least when these structures were accessible to the immunoreagents after sectioning. Such a presynaptic localization might account for the fluorescence associated with the innervated cell surfaces (Fig. 2).

In addition, anti-ankyrin antibody faintly stained the cytoplasm (Fig. 8), and this staining disappeared after preincubation of the anti-ankyrin antibody with purified ankyrin. Indeed, immunoblots of homogenates revealed, in addition to the 200-kD ankyrin band, an immunoreactive polypeptide band of higher molecular weight (>300 kD), which could be a candidate for the cytoplasmic labeling observed.

Discussion

The data presented in this paper on the subcellular localization of proteins from the cytoskeleton in *T. marmorata* electrocyte confirm the asymmetrical organization of this multinucleated cell (21) formerly evidenced by anatomical (40, 51), physiological (56), and cytochemical (8, 9, 59) methods. They further bring possible biochemical bases for the maintenance and even the origin of its polarity.

Innervated Face

Aside from the exclusive presence of the electromotor nerve endings, basal lamina, and synaptic components such as acetylcholinesterase, the innervated face of the electrocyte is characterized by a postsynaptic differentiation of the cytoplasmic membrane, or postsynaptic domain, with a unique biochemical composition.

In addition to the Ach-R polypeptides, present at a particularly high surface density (10), it contains at least one major extrinsic polypeptide: the 43-kD protein (31, 63, 64). In agreement with previous work (45), the m Ab directed against the 43-kD protein exclusively labels the postsynaptic membrane on its cytoplasmic face with, however, interruption at the bottom of the postsynaptic folds where the AchR is also missing (59).

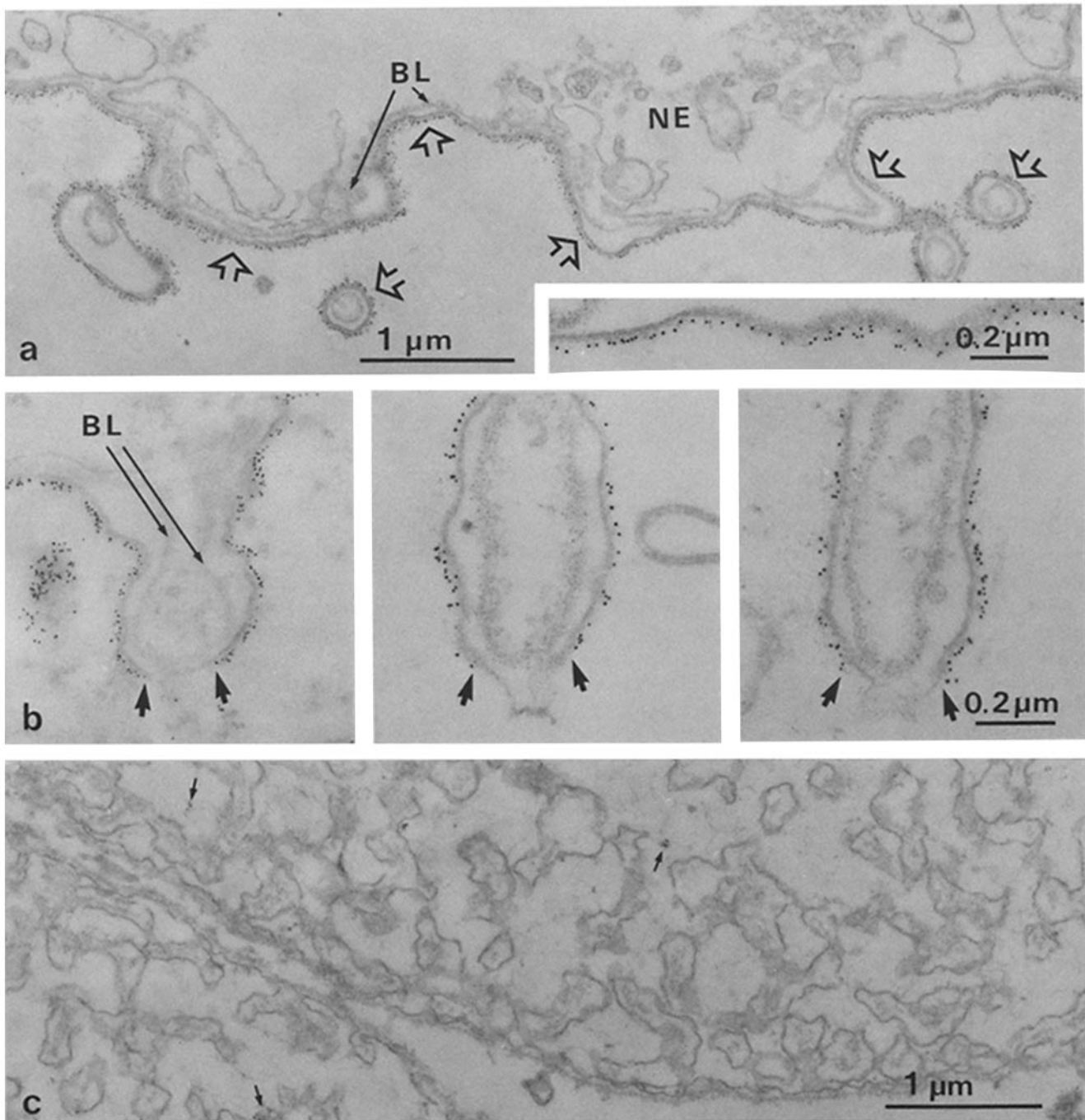


Figure 4. Immunogold localization of the 43-kD protein in electric tissue homogenates. (a) A continuous labeling is observed underlying the cytoplasmic side of an innervated membrane sheet identified by remnants of nerve endings (*NE*) and by the basal lamina (*BL*). (*Inset*) Detail of the gold particle distribution. (b) Interruptions in the labeling are noticed at the bottom of some junctional folds (arrows). (c) The non-innervated membrane fragments, present in the same homogenates and identified by their multiple digitation, were never labeled.

Using a double labeling technique at the electron microscope level, Sealock et al. (61) have demonstrated that the two proteins co-distribute in the membrane. Additional evidence for the close relationships between Ach-R and 43 kD is brought about by cross-linking experiments with purified *T. californica* membrane fragments which disclose a high incidence of cross-linking between the β subunit of the Ach-R and 43 kD (14), suggesting a direct association between the two proteins. On the other hand, reconstitution experiments with purified 43 kD and liposomes make plausible an inter-

action with the lipid bilayer of the postsynaptic membrane (47).

In agreement with the freeze-etching images of Heuser and Salpeter (32), intermediate-sized filaments, which form the bulk of the electrocyte cytoskeleton, terminate onto the cytoplasmic surface of the postsynaptic membrane. The anti-43-kD labeling of the end of some detached intermediate filaments further raises the possibility that these basic components of the cytoskeleton are anchored to the postsynaptic membrane via the 43-kD protein (17).

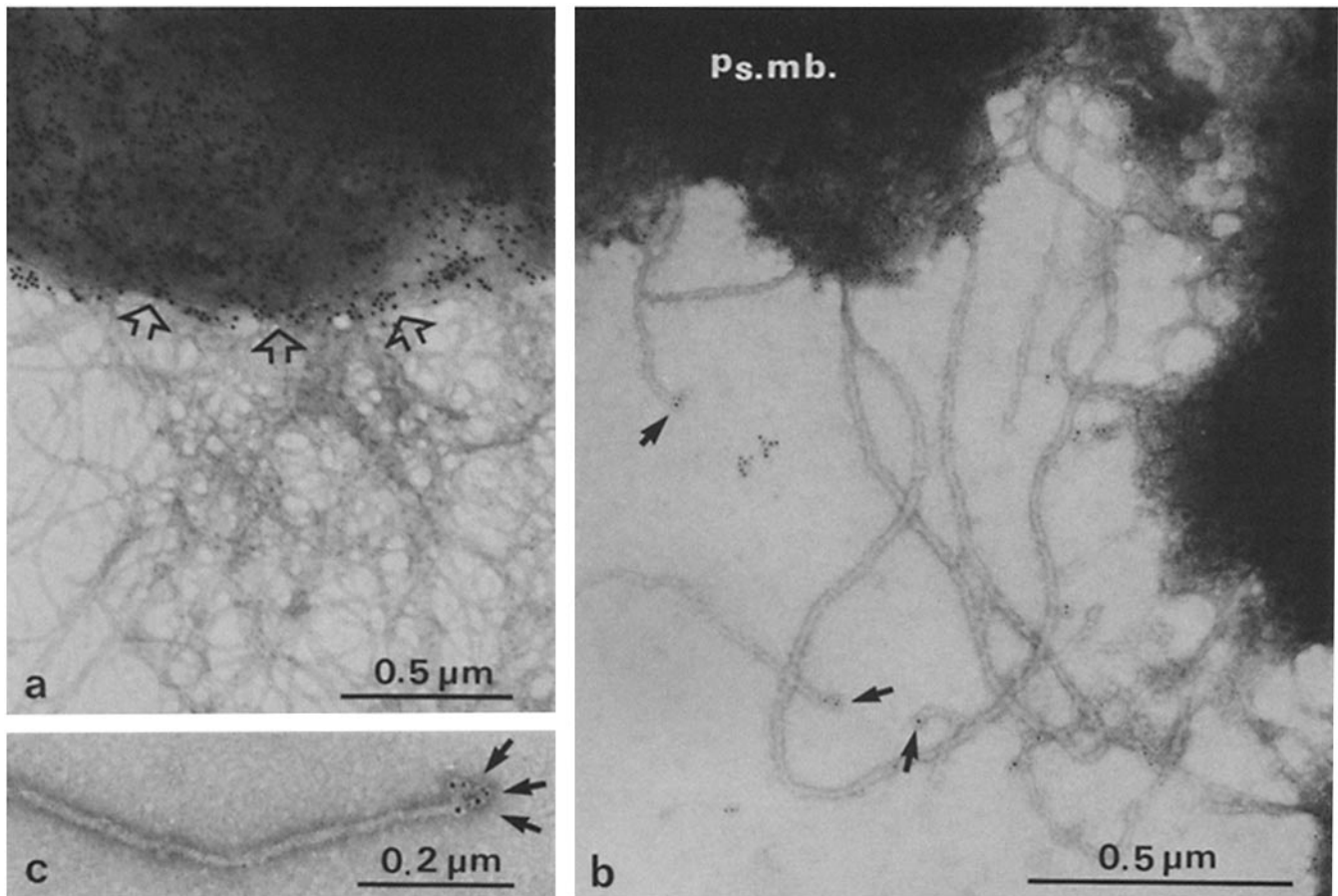


Figure 5. Visualization of the subneural cytoskeleton after negative staining of innervated membrane sheets labeled by anti-43-kD m Ab. (a) A dense network of intermediate-sized filaments is attached to the labeled membrane sheet. Note the absence of labeling within the cytoskeletal network. (b) The ends of some filaments appear labeled (arrows). These filaments are most likely detached from the densely labeled postsynaptic membrane sheet (*Ps.mb.*). (c) Detail of an intermediate-sized filament showing end-on labeling.

Such a simple architecture if valid for the electromotor synapse does not seem sufficient to account for the complex subneural apparatus of the neuromuscular junction. In particular, an additional meshwork of thin, yet unidentified, filaments was observed in the rat beyond the cytoplasmic surface of the junctional folds (33). In addition to molecules immunologically related to the Torpedo 43-kD protein (23, 24), several cytoskeletal components such as a cytoplasmic form of actin (30), talin (60), α -actinin, filamin, vinculin (7), and a 51,000-D protein related to intermediate-sized filaments (12) have been detected by immunofluorescence at the neuromuscular junction. Most of these proteins persisted after denervation and were de facto attributed to the postsynaptic domain.

Since little is known of the molecules that could, in addition to the 43-kD protein, be involved in the molecular architecture of the postsynaptic domain in *Torpedo*, a major objective of the present work was the identification of proteins that could eventually accumulate in this area. The conclusion of our observations is that, despite the detection of several cytoskeletal proteins in the electric tissue (filamin, vinculin, actin) by immunoblotting, none of them were revealed by our techniques at the level of the postsynaptic membrane. Filamin and vinculin were not identified by immunocytochemical methods despite significant cross-reactivity of anti-

filamin and anti-vinculin antibodies with the cytoskeleton-enriched fraction by immunoblotting. The reasons for such discrepancies are not known: masking of the antigenic determinants by steric hindrance, presence of an unreactive native configuration, and/or insufficient local concentrations of the antigens might possibly be invoked. Moreover, the absence of organized actin binding proteins in the innervated membrane corroborates that of actin filaments. Labeling by anti-actin antibodies appeared randomly dispersed into the cytoplasm without any preferential association with the postsynaptic membrane as in the case of the neuromuscular junction (but see below for its redistribution during tissue fractionation). This striking difference might be related to the presence of the complex postsynaptic foldings that characterize the motor endplate but are almost completely absent in the electromotor synapse. In this last instance the 43-kD protein would be the main, if not unique, component directly involved in the immobilization and the anchoring of the Ach-R to the underlying cytoskeleton.

Non-innervated Face

The dorsal non-innervated face of the electrocyte differs from the innervated one by the numerous tubular infoldings of the plasma membrane that contain the Na^+K^+ ATPase. In the present study, we have shown that labeling by antibodies

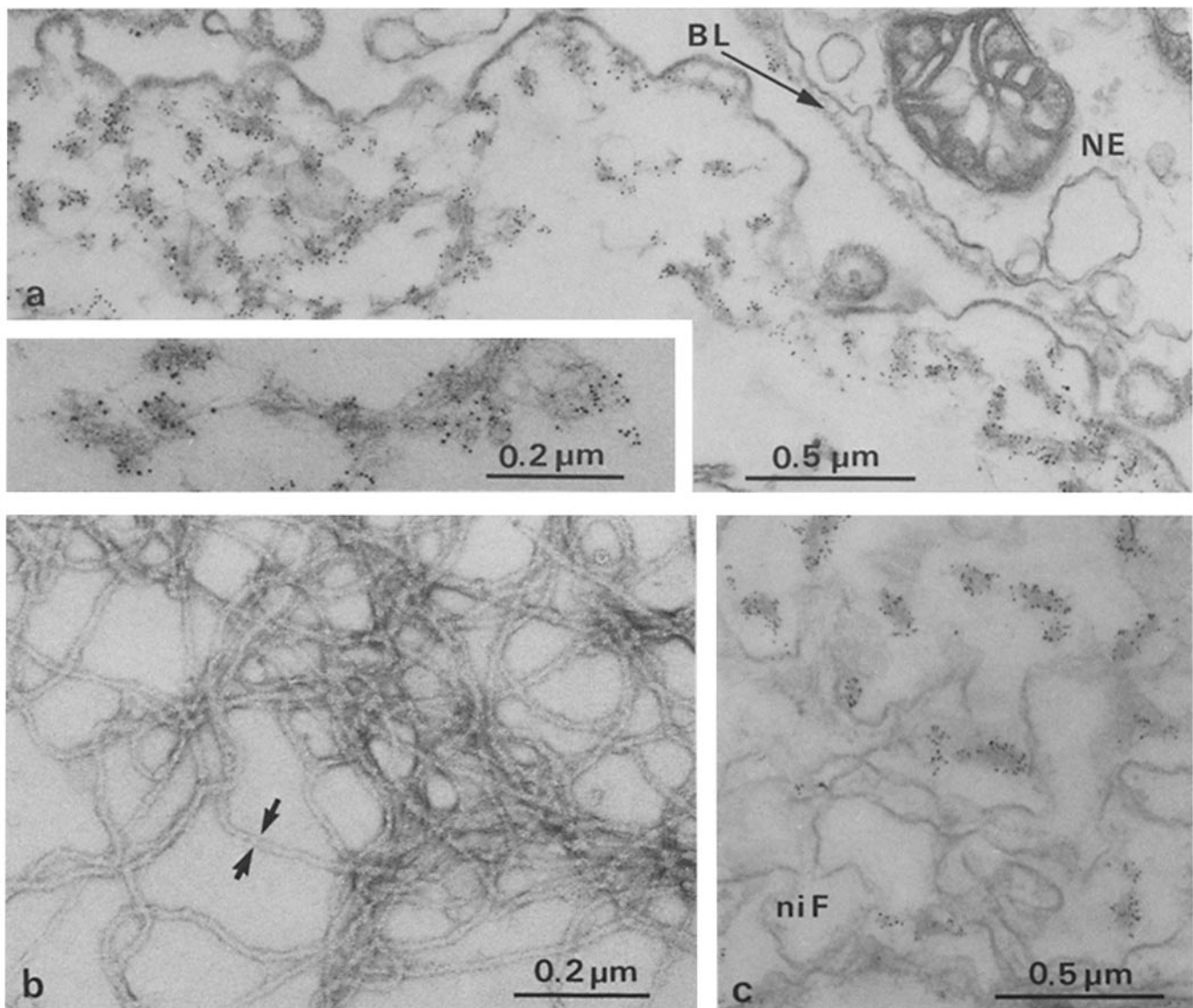


Figure 6. Identification of electrocyte cytoskeletal filaments with the anti-desmin m Ab. (a) Intense labeling of the cytoskeleton network occurs after anti-desmin labeling of electric tissue homogenates. The postsynaptic membrane, as well as other cellular structures, are not labeled. Most of the filaments aggregated during the labeling procedure (staining uranyl acetate/lead citrate). (b) Detailed view of the transcellular network of intermediate-sized filaments (10–12 nm in diameter) (negative staining, 1% uranyl acetate). (c) The non-innervated membrane is also penetrated with the desmin-like protein meshwork (unstained section).

against the two cytoskeletal proteins, fodrin and ankyrin, is preferentially localized in the electrocyte at the level of the infoldings of the non-innervated membrane on their cytoplasmic side. Together with these two proteins, F actin was observed on the non-innervated membrane, making plausible an organization similar to that prevailing in the red blood cell (11). Surprisingly, in *Torpedo* as well as in red blood cell membranes (data not shown), this actin form was not revealed with the anti-actin m Ab which has been demonstrated to recognize both G and F actin forms in various cells (Grimaud, J. A., and D. Paulin, manuscript in preparation). This could result from restricted accessibility of actin in its fodrin-complexed form.

Electron micrographs further show that the faint labeling evidenced by immunofluorescence with anti-ankyrin and anti-fodrin antibodies on the innervated side of the cells is associated with the nerve endings. The presence of fodrin in

that area is not unexpected, since it was found in a variety of tissues, including the central nervous system, where it was first described (28, 37, 38). In addition to ankyrin and fodrin, F actin was also detected within the nerve endings (Kordeli, E., J. Cartaud, H. O. Nghiê, and J. P. Changeux, manuscript in preparation). It is remarkable that these proteins are concentrated within the nerve endings, where important exocytotic activity occurs.

The case of ankyrin is more complex. On the electron micrographs, labeling was found at the level of the non-innervated membrane but also in the cytoplasm. Consistent with its membrane localization is the known function of ankyrin in the erythrocyte as a linkage protein between the cortical cytoskeleton and the plasma membrane (4, 5). On the other hand, the cytoplasmic labeling observed in the electrocyte does not look consistent with this interpretation. Despite its monospecificity in the human red blood cell

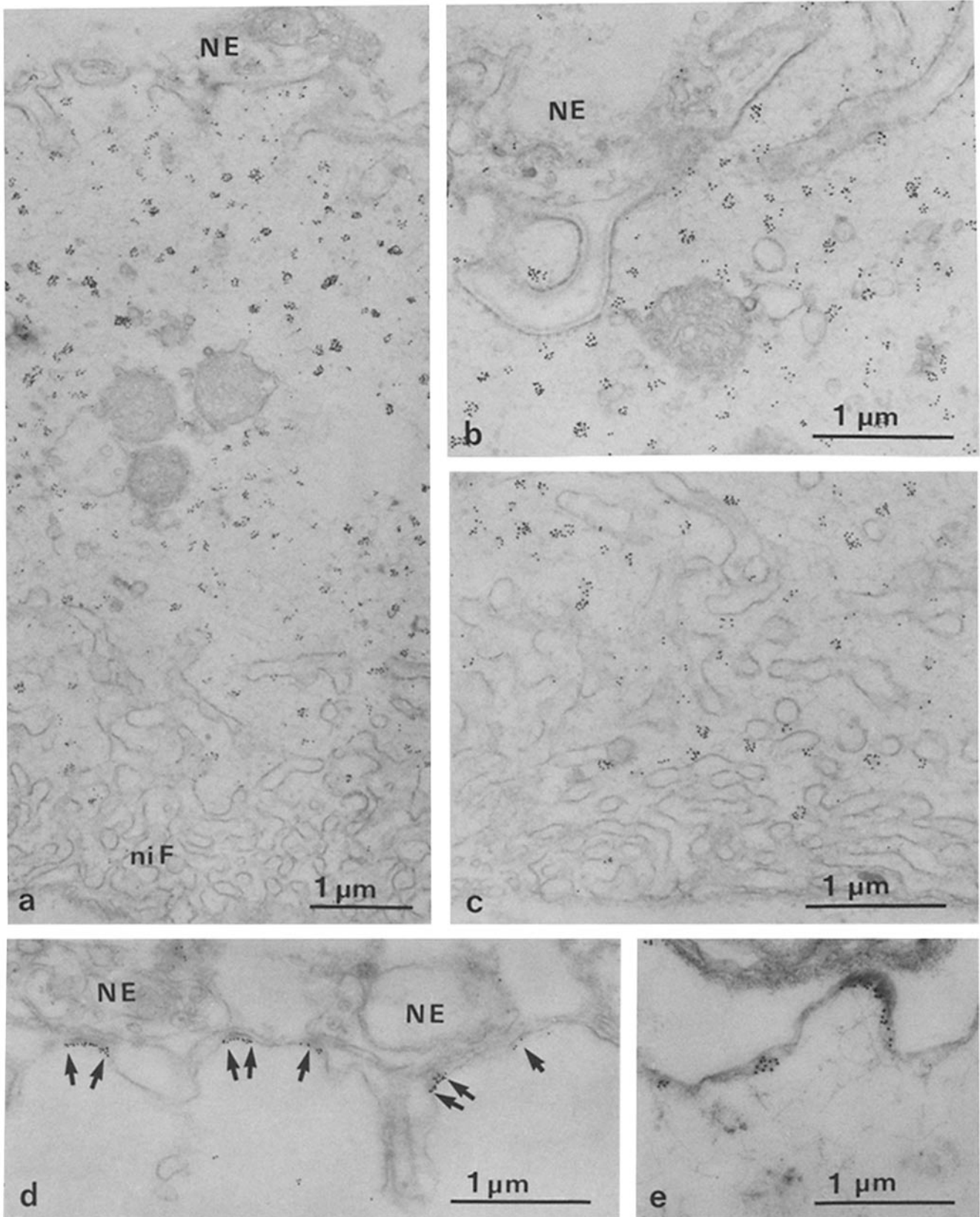


Figure 7. Immunogold localization of actin in *T. marmorata* electrocyte. (*a*, *b*, and *c*) Thin sections through re-embedded cryostat section of paraformaldehyde-fixed tissue. Anti-actin antibodies make rosette-like aggregates scattered throughout the cytoplasm. Membranes and filaments do not appear specifically labeled. The rosette-like aspect does not seem to be caused by artifactual gold aggregation since such images are absent in Figs. 7 and 8, selected from the same set of experiments. *niF*, non-innervated face. (*b* and *c*) Details of the innervated and non-innervated membranes; note the absence of labeling at the membrane level. (*d* and *e*) Anti-actin labeling of membrane fragments derived from *T. marmorata* electric tissue homogenates. Actin actually occurs in patches at the cytoplasmic face of the postsynaptic membrane (*d*, unstained section). Note that the cytoskeleton, still present in these preparations as shown in stained sections, is not labeled (*e*).

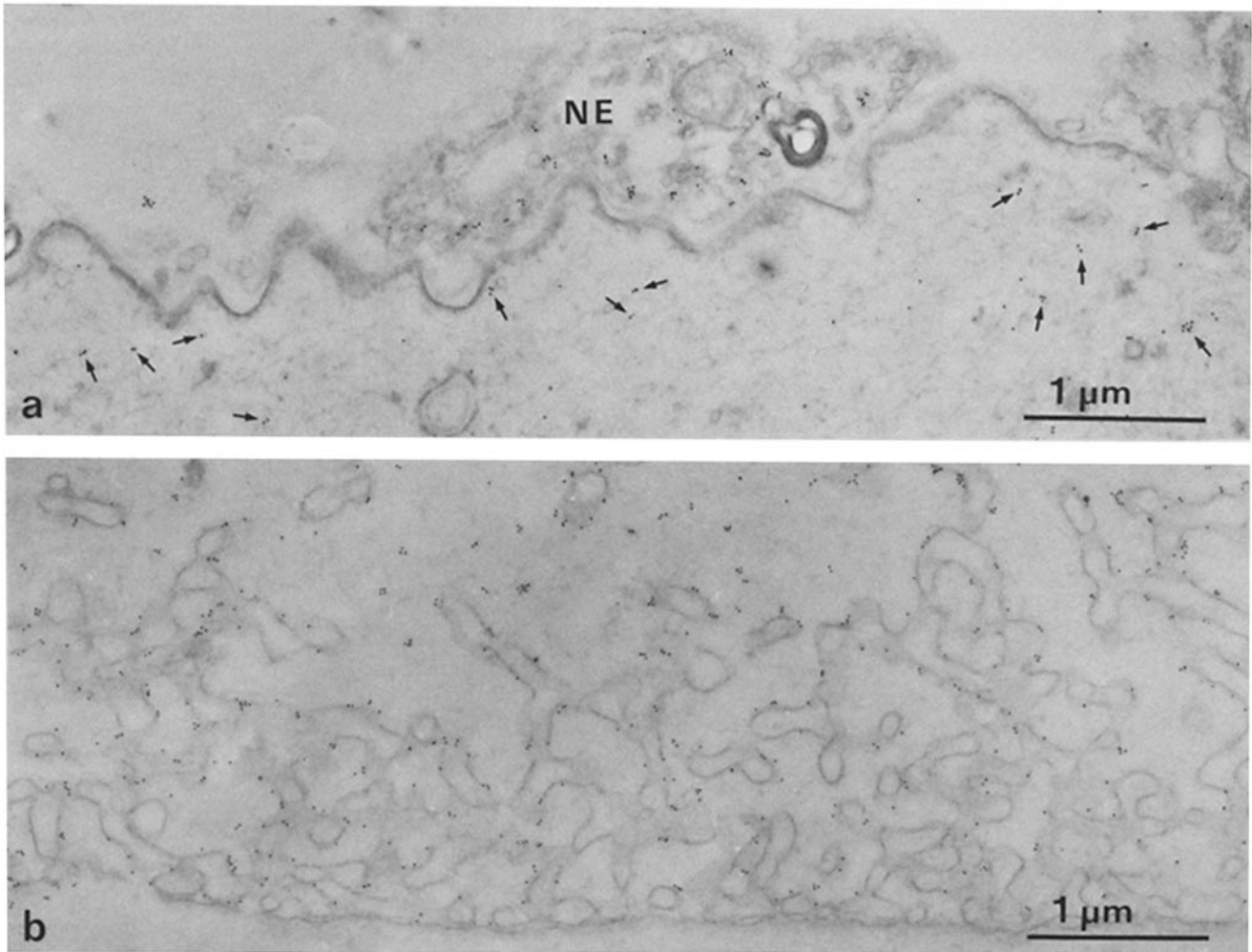


Figure 8. Immunogold localization of ankyrin in *T. marmorata* electrocyte cryostat sections. (a) Ankyrin is not present at the postsynaptic membrane. Nerve endings often appear labeled. Cytoplasmic labeling above background level was also observed (arrows). (b) A dense labeling is consistently observed on the cytoplasmic face of the non-innervated membrane.

(Régnouf, F., E. Nguyen, R. Cassoly, and L. A. Pradel, manuscript in preparation), the antibody used recognizes on immunoblots of electric tissue a band of high apparent molecular weight (≥ 300 kD) in addition to ankyrin (200 kD) which, among several possibilities, might be responsible for the cytoplasmic labeling. This polypeptide may be one of the high molecular weight microtubule-associated proteins that were reported to cross-react with anti-ankyrin antibodies (6).

Transcellular Filament Meshwork

Both anti-desmin and anti-actin antibodies disclose an extensive labeling of the intracellular space. A desmin-like protein was unambiguously associated with the bulk of the filaments that contact both surfaces of the cell, and no immunogold labeling was noticed with an anti-human vimentin m Ab (but vimentin might still be present in fish electric tissue). Thus, in agreement with the muscular embryonic origin of the electric tissue, the intermediate-sized filaments present within *Torpedo* electrocytes essentially contain a desmin-like protein.

Actin has been repeatedly identified in central and peripheral synapses (22, 30, 34, 41) from several species and appears systematically associated with purified postsynaptic membrane preparations from *Torpedo* electric tissue (43,000, ν_3

protein) (29, 46, 66). More recently, a direct *in vitro* interaction between the 43-kD protein and actin was demonstrated by a blotting technique (70) leading to the suggestion of an eventual role of actin in the anchoring of the Ach-R.

Yet, the present data attesting of a strict cytoplasmic distribution of G actin and absence of membrane-bound F actin on the postsynaptic membrane in electrocyte fixed tissue sections do not support this view. At variance, parallel immunogold experiments carried out with unfixed homogenates of electric tissue disclose a different pattern with an unambiguous staining of the cytoplasmic face of the postsynaptic membrane fragments, whereas cytoplasmic labeling has disappeared.

In agreement with these observations, Strader et al. (66) reported that the major part of electrocyte actin was recovered in the supernatant fraction in a form that inhibits DNase I (G actin), while a minor portion exhibiting the same inhibition property was associated with the postsynaptic membrane fragments. Taken together, these data led to the proposal that membrane-bound actin in electrocyte homogenates results from a redistribution of a soluble G actin pool. The *in vitro* interaction between actin and the 43-kD protein reported by Walker et al. (70) would then reflect functionally unrelated

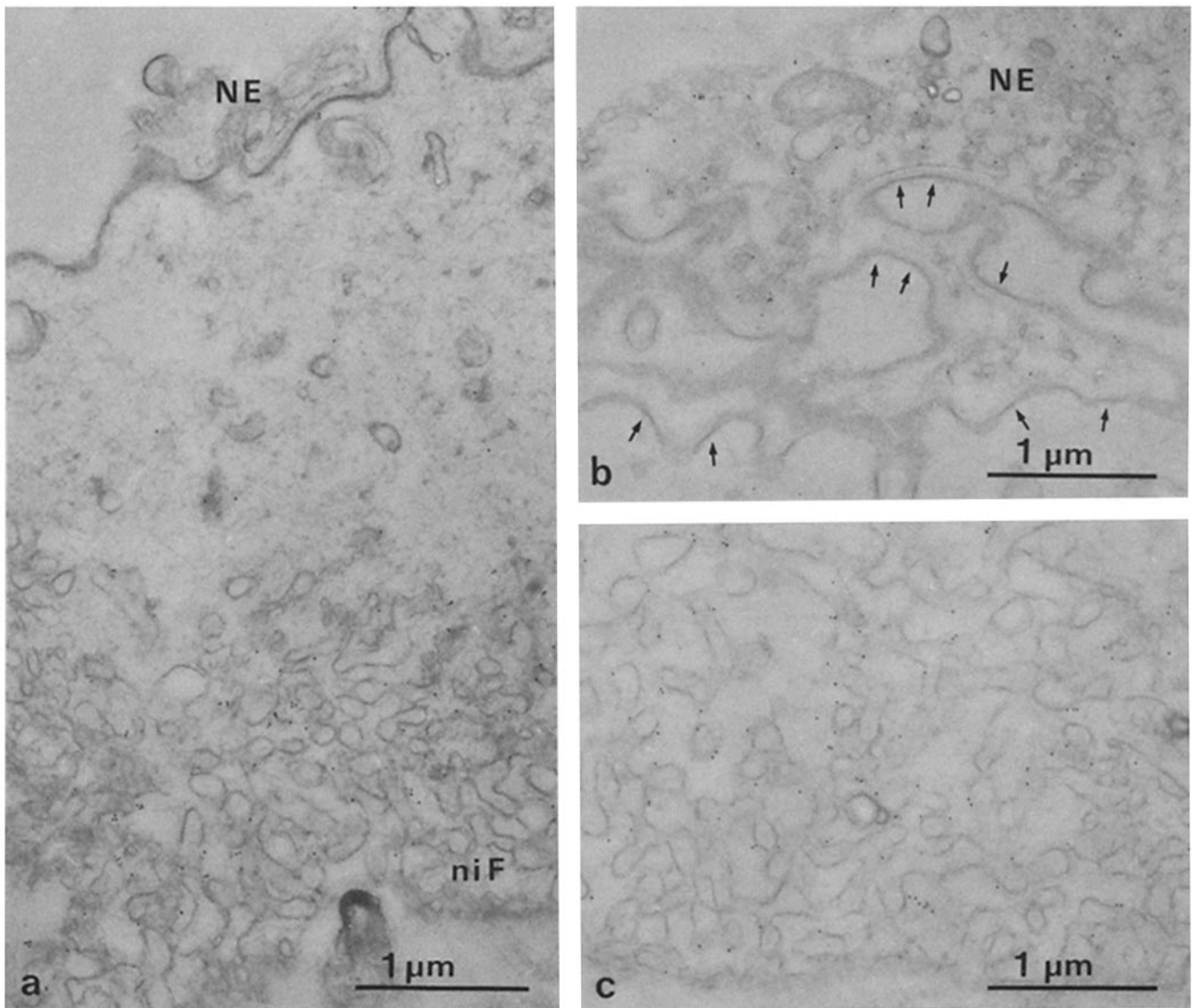


Figure 9. Immunogold localization of fodrin in *T. marmorata* electrocyte cryostat sections. (a) A thin section throughout the cell discloses a predominant labeling of the non-innervated face. (b) Detailed view of a synaptic area confirms the absence of fodrin from the postsynaptic membrane (arrows). However, nerve endings are often labeled. (c) Detailed view of the non-innervated area. The gold granules occur on the cytoplasmic face of the plasma membrane infoldings.

adhesion properties between these two proteins. However, one cannot rule out that a complexed actin associated with the postsynaptic membrane remained undetected in situ and became unmasked upon tissue fractionation.

In conclusion, we have identified within the electrocyte an anisotropic arrangement of cytoskeletal proteins that co-distribute respectively with the innervated Ach-R-rich, the non-innervated ATPase-rich domains of the plasma membrane, and the transcellular cytoplasmic meshwork. In the postsynaptic domain, the 43-kD protein is the unique cytoplasmic component yet identified that could contribute to Ach-R clustering and anchoring to the underlying cytoskeleton. On the other hand, anti-ankyrin and anti-fodrin antibodies, as well as F actin, are localized exclusively at the ATPase-rich, non-innervated side where, as in the erythrocyte, they might be engaged in the association of intrinsic proteins of the membrane with the cytoskeleton. Desmin is a plausible component of the transcellular meshwork that links the two faces

of the cell and maintains its polarity. The anti-actin antibody staining is consistent with a large cytoplasmic G actin pool not directly associated in situ either with membranes or with filaments.

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