

Subcellular Localization of Creatine Kinase in *Torpedo* Electrocytes: Association with Acetylcholine Receptor-rich Membranes

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ABSTRACT Creatine kinase (CK, EC 2.7.3.2) has recently been identified as the intermediate isoelectric point species (pI 6.5–6.8) of the M_r 40,000–43,000 nonreceptor, peripheral ν -proteins in *Torpedo marmorata* acetylcholine receptor-rich membranes (Barrantes, F. J., G. Mieskes, and T. Wallimann, 1983, *Proc. Natl. Acad. Sci. USA*, 80: 5440–5444). In the present study, this finding is substantiated at the cellular and subcellular level of the *T. marmorata* electric organ by immunofluorescence and by protein A-gold labeling of either ultrathin cryosections of electrocytes or purified receptor-membrane vesicles that use subunit-specific anti-chicken creatine kinase antibodies. The muscle form of the kinase, on the one hand, is present throughout the entire *T. marmorata* electrocyte except in the nuclei. The brain form of the kinase, on the other hand, is predominantly located on the ventral, innervated face of the electrocyte, where it is closely associated with both surfaces of the postsynaptic membrane, and secondarily in the synaptic vesicles at the presynaptic terminal. Labeling of the noninnervated dorsal membrane is observed at the invaginated sac system. In the case of purified acetylcholine receptor-rich membranes, antibodies specific for chicken B-CK label only one face of the isolated vesicles. No immunoreaction is observed with anti-chicken M-CK antibodies. A discussion follows on the possible implications of these localizations of creatine kinase in connection with the function of the acetylcholine receptor at the postsynaptic membrane, the Na/K ATPase at the dorsal electrocyte membrane, and the ATP-dependent transmitter release at the nerve ending.

Creatine kinase (CK)¹ as an ATP-regenerating system is thought to play an important role in the energy metabolism of skeletal and heart muscle as well as in brain (11, 14, 39). This enzyme is crucially involved in maintaining adequate intracellular ATP/ADP ratios and phosphoryl-creatine pool sizes (for review, see reference 8). In muscle, relatively small amounts of total CK—which in the past has been considered

as an entirely soluble enzyme—are specifically associated with intracellular “compartments,” such as the inner mitochondrial membrane (21, 32), myofibrils (37, 40, 42), sarcoplasmic reticulum (6, 23), and plasma membrane (17, 34). These facts have led to the proposal of the phosphoryl-creatine shuttle model (8, 38, 39, 41), which implies that CK is functionally coupled at sites of energy production to ATP-generating systems (e.g., mitochondria and cytosol glycolysis), and at sites of energy consumption to ATP-utilizing systems (e.g., myofibrillar actin-activated Mg^{2+} -ATPase, Ca^{2+} ATPase, and Na/K-ATPase).

The nonreceptor, peripheral ν -proteins (so-called 43K-proteins, M_r 40,000–43,000) are conspicuous components of the

¹ Abbreviations used in this paper: AChR, acetylcholine receptor; B-CK, brain-type creatine kinase; GarG, goat anti-rabbit IgG gold-conjugate; M-CK, muscle-type creatine kinase; PBG, phosphate-buffered saline containing 0.2% gelatin and 0.5% bovine serum albumin.

acetylcholine receptor (AChR)-rich membranes and the *Torpedo marmorata* electrocyte (for review, see reference 3). At least some of these proteins are also found in the mammalian neuromuscular junction (15). It is becoming increasingly apparent, however, that they constitute a collection of proteins whose only common property is their similar molecular weight, isoelectric focusing already making evident their marked charge heterogeneity (18). In terms of function, it has only recently been possible to identify the enzymatic activity of creatine kinase in distinct members of the ν -proteins, i.e., in the intermediate pI (6.5–6.8) ν_2 -species (4, 5). Protein kinase activity has also been reported to be associated with the ν -proteins (16), but it is not known which of these polypeptides displays the enzymatic activity.

Although both "muscle"-type (M) and "brain"-type (B) CK isoenzymes are found in electric tissue, only the latter type appears to be associated with purified AChR-rich membranes on the basis of biochemical and immunochemical criteria obtained with anti-chicken CK antibodies (4, 5). The nature of this association is not known in this and other ν -proteins, of which the more basic, ν_1 -species exhibit the strongest binding. Though the extractability from native membranes of all species at low ionic strength/alkaline pH would suggest ionic interactions with the membrane constituent, the inability of this treatment to deplete ν_1 and ν_2 from membranes stabilized *ab initio* by *N*-ethylmaleimide alkylation (1, 4) suggests the participation of some other types of forces in which thiol groups may be involved. The biochemical definition of "membrane-bound" as well as "cytosol" forms of the ν -proteins is also operational, depending on the technique of membrane purification (e.g., affinity partitioning, gradient centrifugation, countercurrent distribution; see reference 18).

In the present study, the association of one of the CK isoenzymes with the electrocyte plasmalemma is demonstrated *in situ* by immunocytochemical techniques. As was found earlier in differentiating myogenic cells (40), the CK isoenzymes are located in *T. marmorata* electrocytes in an isoenzyme-specific manner at different intracellular locations. The mostly soluble M-CK, on the one hand, is present throughout the entire electrocyte with the exception of the nuclei. B-CK, on the other hand, is found associated with the dorsal membrane sacs, the postsynaptic membrane, and the synaptic vesicles in the presynaptic terminal. In addition, it has been possible to localize B-CK directly on purified isolated AChR-rich vesicles, where it appears predominantly on only one face of the membrane. Part of this work has been presented in abstract form (43).

MATERIALS AND METHODS

Materials: Native α -bungarotoxin was from the Miami Serpentarium, Miami, and its tritium derivative from Amersham Buchler, Braunschweig, Federal Republic of Germany. *T. marmorata* electric fish were obtained from the Marine Biological Station at Arcachon, France, and from the Pharmacology Department, University of Zürich.

Preparation of AChR-rich Membranes: Membranes from the electric tissue of *T. marmorata* were prepared by the procedure given in reference 2. Typical specific activities of 2–4 nmol α -toxin sites/mg of protein were obtained. The membranes were stored in liquid N_2 until further use. Storage did not alter their morphological characteristics; most AChR-rich membranes appear as sealed, right-side-out vesicles as judged by electron microscopy and flux experiments (for references, see review in reference 3). The sidedness of the AChR membranes was assayed as reported by Hartig and Raftery (20).

Immunolabeling of Paraffin-embedded Material: Single electrocyte columns were dissected from the electric organ, fixed overnight with 3% paraformaldehyde in PBS at 4°C, dehydrated through a graded series of

ethanol (70% ethanol for 4 h, 80%, 90%, and 100% ethanol with two changes each for 1 h), transferred to 100% xylene (two changes for 45 min each), and penetrated with paraffin (Paraplast tissue-embedding medium, Lancer, Sherwood Materials, Ireland) twice for 45 min at 60°C. The embedded material was solidified at room temperature and stored in the cold. 5- μ m sections were cut, floated onto 45°C water, and picked up with glass slides previously covered by a thin layer of chrome-alum gelatin (0.1 g of ammonium chromate, 1 g of gelatin in 100 ml of water). Paraffin was dissolved by two 10-min dips in xylene, and the sections were rehydrated through a graded series of ethanol (100, 96, 90, 70, and 30% for 10 min each) and washed with PBS (three changes, 15 min each). Sections were incubated in a moist chamber with specific rabbit anti-chicken B-CK or with anti-chicken M-CK antibodies diluted to 1–5 μ g/ml of specific IgG into PBS-buffer containing 1 mg/ml of bovine serum albumin. The antisera or affinity-purified antibodies used were specific for the B and M subunits of chicken and crossreacted with *T. marmorata* CK as has been shown previously (4, 5). The antibodies have been extensively characterized in the homologous chicken system (10, 27, 36, 40, 42). Preimmune sera or corresponding rabbit IgG were used at the same dilutions as in the controls. After incubation for 30 min at 25°C followed by three washings with PBS, 100–200 μ l of 1:100 diluted fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA) were placed on the same area, and the sections were incubated for another 30 min. After three washings with PBS and removal of excess PBS, a drop of 50% glycerol in 0.1 M glycine-NaOH at pH 9.0 was placed onto the sections which were then covered by a coverslip, examined with a Zeiss standard-18 epi-fluorescence microscope (Carl Zeiss Inc.), and photographed on Ilford HP-5 film (Ilford Ltd., Ilford, Essex, England).

Cryosections for Light Microscopy: Dissected electrocyte columns were cut perpendicularly to their longitudinal axis into small pieces, immersed into Tissue Tek II embedding medium (Lab-Tek Products, Miles Laboratories Inc., Elkhart, IN), and immediately frozen by CO_2 evaporation. 20- μ m sections were cut in a Cryo-cut microtome (American Optical Corp., Instrument Div., Buffalo, NY) and picked up with chrome-alum gelatin-coated microscope slides. After most of the soluble MM-CK was washed out with PBS for 10–20 min the membrane-bound B-CK was visualized by indirect immunofluorescence staining as described above.

Ultracytosectioning and Immunoelectron Microscopic Histochemistry: Freshly excised electric organ was cut into small segments of ~2 mm³ and fixed for 2 h with 2% paraformaldehyde and 0.2% glutaraldehyde in ice-cold PBS at pH 7.4. The mildly fixed tissue pieces were infused for 2 h with 1.15 M sucrose in PBS and then left overnight at 4°C in PBS containing 2.3 M sucrose (35). The tissue segments were then placed on specimen supports, consisting of short copper rods with a flattened end, and frozen by direct immersion into liquid nitrogen. Ultrathin sections were cut at –120°C with an Ultracut (Reichert-Jung, Vienna, Austria) fitted with a Cryokit FC4. The sections were picked up with a droplet of 2.3 M sucrose in PBS and mounted on collodion-carbon-coated copper grids. To wash away the sucrose, the grids were placed facedown on a buffer surface (0.1 M glycine in PBS at pH 7.4). The immunocytochemical reaction was based on the method described by Roth et al. (30). However, instead of protein A-gold, a goat anti-rabbit IgG gold-conjugate (GarG, Janssen Pharmaceutica, Beerse, Belgium) with a gold particle size of ~5 nm was used. Rabbit anti-chicken B-CK antibody purified by affinity chromatography and corresponding nonimmune IgG were used at concentrations of 1–10 μ g/ml as specified in the figure legends. All steps of the immunolabeling were performed on 100- μ l drops arranged on sheets of paraffin. Grids were first floated for 10 min onto 0.1 M phosphate buffer containing 50 mM glycine, pH 7.4. To minimize unspecific labeling, the grids were transferred twice for 10 min each on droplets of PBS containing 0.2% gelatin and 0.5% bovine serum albumin, pH 7.4 (referred to as PBG). After incubation for 2 h at 20°C with anti-B-CK IgG or control IgG diluted with PBG, the sections were transferred through a series of several drops of PBG to wash away unbound antibodies.

The sections were then incubated for 1 h at 20°C with GarG diluted 1:100 with PBG. After extensive washing with PBG, with 0.1 M glycine in PBS, and finally water, the sections were stained for 10 min first with neutral and then for 2 min with acidic 1% uranyl acetate. Mechanical support for the sections was provided by allowing them to dry in a 1.3% aqueous solution of methyl cellulose. Sections were examined with a Philips 301 electron microscope (Philips Electronic Instruments, Inc.).

Immunocytochemistry on Isolated AChR-rich Membrane Vesicles: Colloidal gold with particle diameters of 2–5 nm obtained by reduction with sodium borohydride was bound to protein A as described (29) to give protein A-gold (kindly provided by M. Bähler, Inst. Cell Biol.). AChR membrane vesicles were diluted with PBS to a final protein concentration of 0.1–0.2 mg/ml and adsorbed for 1–2 min onto grids previously coated with carbon by glow discharge. In a first set of experiments the adsorbed vesicles were incubated initially with PBS containing 0.5% bovine serum albumin for

10–15 min and then either with affinity purified anti-chicken B-CK antibody diluted to 0.5–5 µg/ml or with nonimmune IgG diluted to the same concentration with the above buffer. After extensive washing (10–15 times) with PBS for 1 h, the vesicles, still adsorbed to the grids, were incubated for an additional h with protein A-gold diluted with PBS to 1:100 or 1:200 immediately before use. After extensive washing (10–15 times) with PBS and finally with distilled water, the grids were blotted with filter paper and either negatively stained with 1% uranyl acetate in H₂O or quick-frozen in supercooled liquid nitrogen and then freeze-dried (see below).

In a second set of experiments, the grids with the adsorbed vesicles were squirted with a stream of PBS to break open the vesicles and to expose both the inner and outer membrane surfaces to the antibodies (9, 25). After the squirting procedure, the vesicles were fixed for 15 min at 20°C with 3% paraformaldehyde–0.1% glutaraldehyde in PBS. After fixation, vesicles were washed several times with PBS and quenched for 15 min with PBS containing 0.1 M glycine. After extensive washing the vesicles remaining on the grids were either processed directly for immunolabeling as described above or incubated for 1 min each in PBS containing 0.1% SDS, washed with PBS, and followed by incubation in PBS containing 0.1% Triton X-100 (to expose antigenic sites of B-CK, which might otherwise not have been available on the native membranes). Immunolabeling of the vesicles subjected to these various treatments was performed as described above. As an additional control, the first antibody was omitted and the vesicles incubated with protein A-gold only.

For freeze-drying, the vesicles remaining on the carbon grids were inserted while still liquid nitrogen-frozen into a precooled magnetic table (45), which in turn was transferred via a counterflow loading device onto the precooled rotary stage (Balzers BAF 300) at –80°C in vacuo ($P \leq 5 \times 10^{-7}$ mbar). The specimens were unidirectionally shadowed at a 45° angle with 0.2 nm of Pt/C, subsequently backed with 5–10 nm of C, slowly warmed to room temperature, and finally withdrawn. Specimens were examined in a JEOL JEM 100 C electron microscope equipped with an anticontamination device at an acceleration voltage of 100 kV. Pictures were taken at $\times 50,000$ on AGFA-Gevaert Scientia film. The magnification was calibrated using catalase crystals as a reference. The negatives were used directly to produce enlarged positive prints (heavy metal deposits are dark, shadows are white).

Other Analytical Procedures: Protein concentration of the membranes was determined by the procedure of Lowry et al. (24), using bovine serum albumin as standard. The specific activity of the membranes in terms of α -toxin binding capacity was assayed as in reference 2. The sidedness of the AChR-rich membranes was determined according to reference 20.

RESULTS

Indirect immunofluorescence staining with anti-chicken B-CK antibody of paraffin embedded electrocytes or cryosectioned stacks of electrocytes from *T. marmorata* leads to bright fluorescence of the electrocyte surface membranes, especially the ventral innervated faces of the electrocyte where the acetylcholine receptor is located (Fig. 1*b*). Staining with anti-B-CK antibody at the noninnervated dorsal membrane was weaker and somewhat more diffusely distributed over the entire area of the dorsal face (Fig. 1*b*). Incubation with anti-chicken M-CK antibodies, on the contrary, led to a bright staining of the whole electrocyte except the nuclei (Fig. 1*d*). Incubation with control antibody led to very faint background fluorescence (Fig. 1*f*).

M-CK, distributed over the entire electrocyte with the sole exception of the nuclei, seems to be a mainly soluble CK isoenzyme. This may be inferred from the fact that prolonged washing of unfixed cryosections of electric organ before indirect immunofluorescence staining with anti-M-CK antibody led to a significant decrease in the fluorescence intensity of these cells (not shown). Using the same argument, the B isoform appears to be tightly associated with the membrane system of the electrocytes in that prolonged washing with physiological buffers or even a short incubation with PBS containing 0.1% Triton X-100 did not significantly alter the intensity of the anti-B immunofluorescence staining (Fig. 2*b*). The results demonstrate the isoenzyme-specific localization or intracellular segregation of CK isoenzymes in the *T. marmorata* electrocyte.

Incubation of ultrathin cryosections of mildly prefixed electroplaque tissue with specific anti-B-CK antibody followed by gold-conjugated second antibody led to labeling of the membrane-sac system at the dorsal face of the electroplaques (Fig. 3*a*) and to heavy labeling of the postsynaptic membrane at their ventral face (Fig. 3*c*). In addition, labeling of the synaptic vesicles in the presynaptic nerve endings was seen regularly (*inset*, Fig. 3*c*). Unspecific labeling with protein A-gold seen in the corresponding sections after incubation under conditions identical to those of control IgG, followed by protein A-gold, was insignificant (Fig. 3, *b* and *d*). This method did not suffice to determine unambiguously the sidedness of antibody labeling at the postsynaptic membrane in that immunogold particles were seen on both sides of the rather thick postsynaptic membrane complex.

When isolated AChR-rich membrane vesicles were incubated with anti-B-CK antibody followed by protein A-gold, and then adsorbed onto grids previously coated with carbon by glow discharge, a one-sided labeling was apparent in those membrane vesicles partially disrupted by the adsorption and freeze-drying process (Fig. 4*a*). The outer surface (*os*) of the membranes (dark steps: heavy metal deposit after unidirectional shadowing that indicates one step up) showed fairly even distribution of gold particles with occasional formation of clusters, whereas the inner surface (*is*) of the vesicles (white contours and white steps after unidirectional shadowing that indicate one step down) showed very few gold particles or none at all (Fig. 4*a*). The fact that the outside of the vesicles is accessible to anti-B-CK antibody is indicated further by labeling of almost intact AChR-rich membrane vesicles which were negatively stained instead of freeze-dried (Fig. 4*c*). Incubation with identical or higher concentrations of control IgG followed by protein A-gold led only to insignificant labeling of the vesicles (Fig. 4*b*). Even though the rosettelike particles identified as the AChR protein (see review in reference 3) could not be seen directly after heavy metal shadowing of the vesicles, and though this technique could not be used to discriminate the fine structural details of the inner and outer surface of the vesicles, it is obvious from negatively contrasted vesicles of the same preparation that they are indeed heavily enriched in AChR particles evenly distributed or clustered throughout (Fig. 4*d*, *inset*).

To expose the presumptive inner surface of the AChR membranes directly to the antibody, the vesicles were first opened deliberately by a jet stream of buffer and then incubated with anti-B-CK IgG followed by protein A-gold. By this method only the presumptive outer surface (*os*) of the vesicles was labeled while little gold was deposited on the presumptive inner surface (*is*) (Fig. 5, *a* and *b*). This was also true if vesicles were prefixed before immunolabeling (not shown) or prefixed and subsequently treated with SDS and Triton X-100 with a view to expose hidden antigenic determinants of the membrane-bound B-CK to the antibody (Fig. 5*a*). One-sided labeling was also seen in partially opened but unfixed vesicles (Fig. 5*b*). Incubation before protein A-gold treatment with identical or higher concentration of control IgG (Fig. 5, *c* and *d*) or PBS only (Fig. 5, *e* and *f*) led to insignificant unspecific labeling of the vesicles and did not reveal unspecific binding of protein A-gold itself on either side of the AChR-rich membrane vesicles. These results demonstrate an asymmetrical distribution of B-CK on the AChR-rich membrane; the association appears to be circumscribed to their presumptive outer surface (or at least the outer surface as the vesicle lies on the grid). In addition, B-CK also appears to be associated

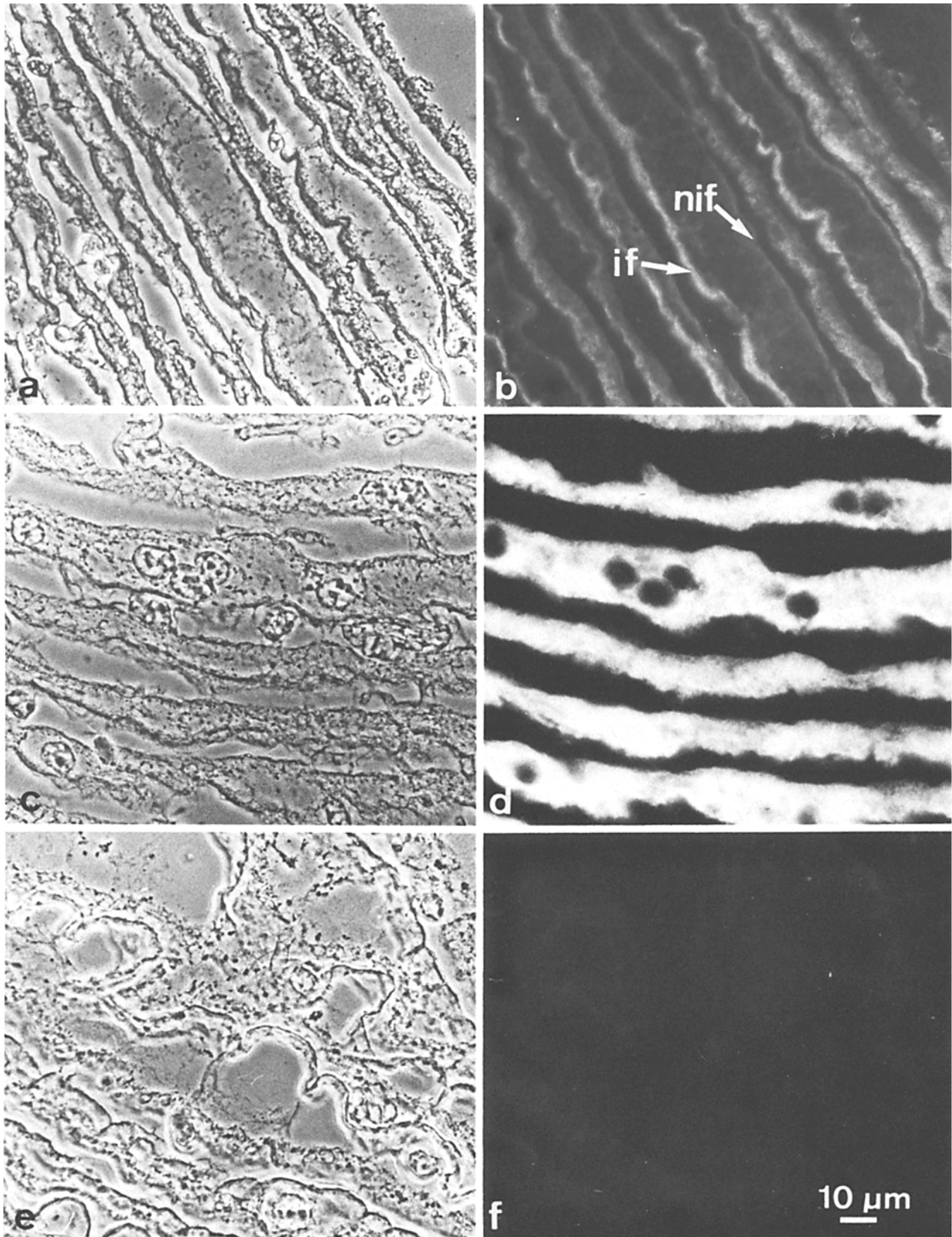


FIGURE 1 Immunofluorescence staining of paraffin-embedded electric organ. Sections of paraffin-embedded electric organ from *T. marmorata* stained by indirect immunofluorescence for B-CK (a and b) and for M-CK (c and d) using specific rabbit anti-chicken B- and M-CK antibodies, respectively. Controls incubated with the same concentration of preimmune IgG (e and f) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as second antibody. Phase-contrast (a, c, and e) and immunofluorescence (b, d, and f) pictures. Note the bright fluorescence after staining for B-CK at the membranes, especially the ventral, innervated face of the electrocyte stacks (b) and the intense overall staining after incubation with anti-M-CK antibody except in the nuclei (d). *if*, innervated face; *nif*, non-innervated face. Bars, 10 μ m. \times 650.

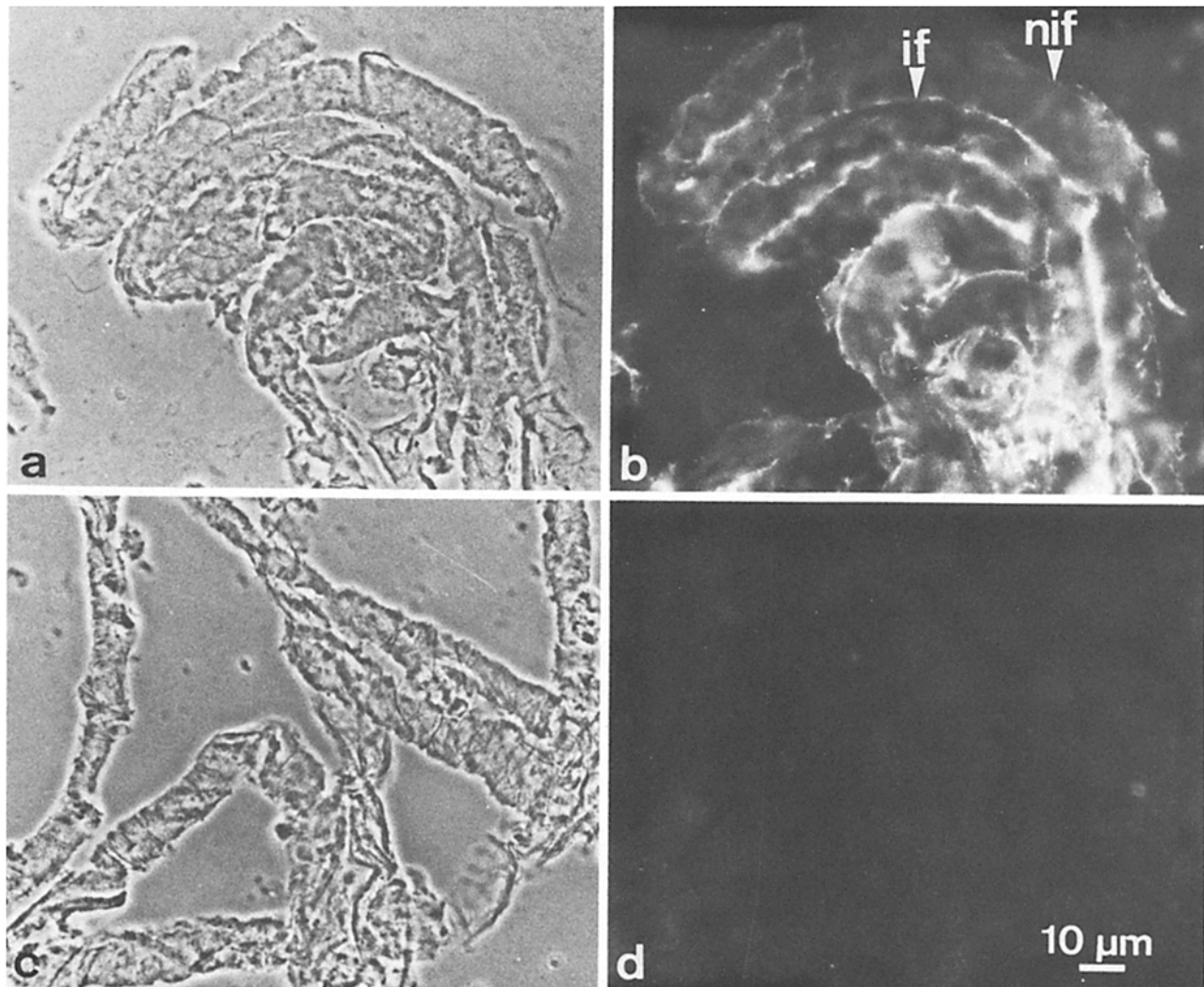


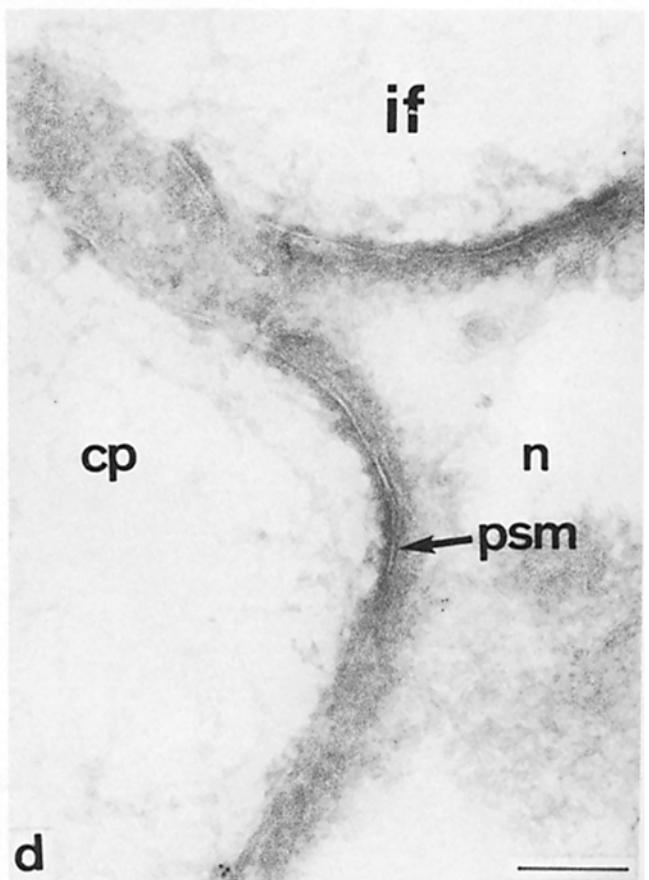
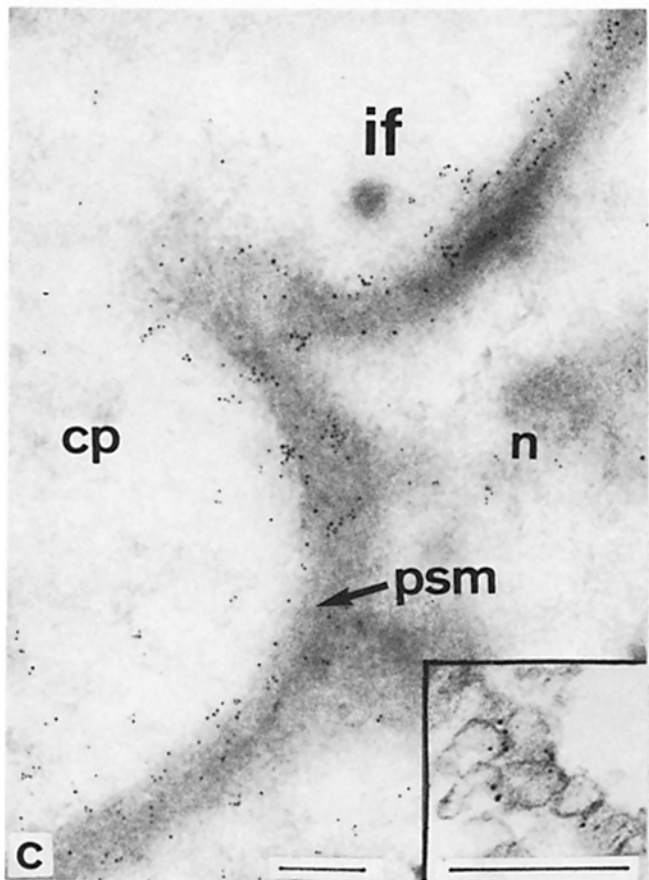
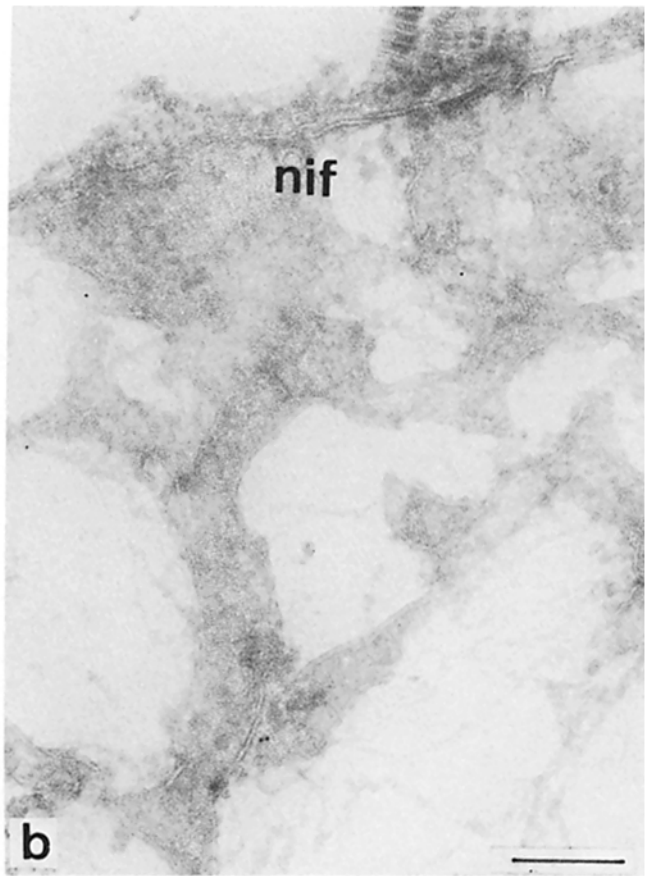
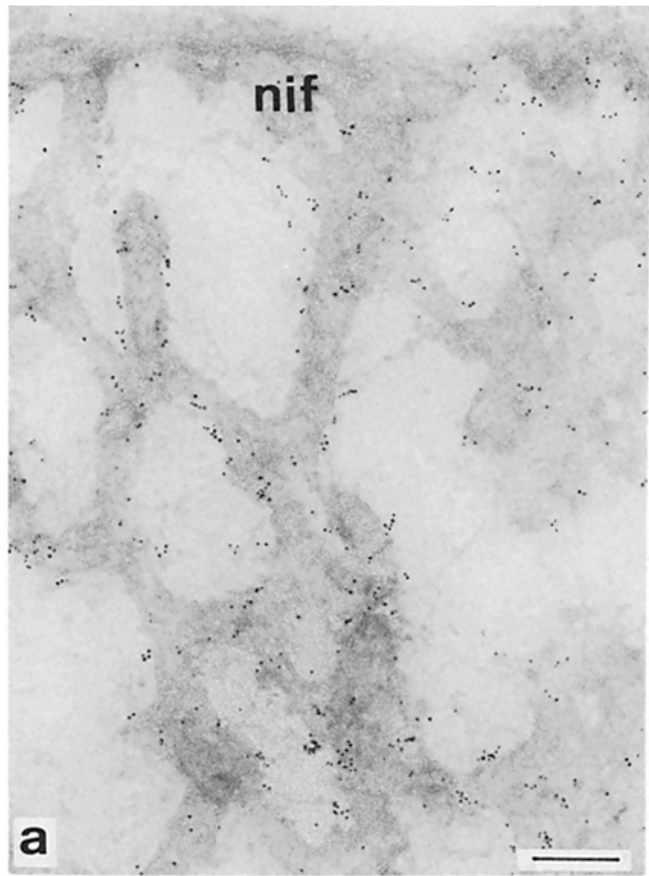
FIGURE 2 Immunofluorescence staining of cryosectioned electric organ. Cryosections of electric organ from *T. marmorata* stained by indirect immunofluorescence for the B-form of CK after extensive washing with PBS (a and b); controls after incubation with preimmune IgG (c and d); both followed by fluorescein isothiocyanate-conjugated second antibody. Phase-contrast (a and c); fluorescence pictures (b and d). Note the bright fluorescence at the ventral, innervated membranes and the weaker, more diffuse staining of the dorsal membrane system of the electrocyte stacks after staining for B-CK (b). *if*, innervated face; *nif*, non-innervated face. Bars, 10 μm . $\times 650$.

with synaptic vesicles at the presynaptic nerve ending and with the dorsal membrane system at the noninnervated electrocyte face.

DISCUSSION

Electric organ, like skeletal muscle, heart, and brain, is a tissue of high energy expenditure, and it is therefore not surprising to find some CK content. As in the case of muscle, where compartmentalization of CK isoenzymes has been demonstrated (40), an isoenzyme-specific segregation of CK isoenzymes seems to exist within the *T. marmorata* electrocyte. The present study shows that the muscle type of enzyme, M-CK, on the one hand, is distributed throughout the cell except in the nuclei. Electrocyte B-CK, on the other hand, seems to be associated with the innervated, ventral postsynaptic membrane and the noninnervated dorsal membrane. B-CK has been shown to represent a minor component of the nonreceptor, peripheral ν -proteins in AChR-rich vesicles derived from the former membrane (4).

The finding of creatine kinase in *Torpedo* electrocytes has recently been confirmed by Gysin et al. (19). However, there is disagreement as to which of the CK isoenzymes is being referred to. Our evidence for B-type CK being associated with the receptor membranes is based first on cellulose polyacetate electrophoresis of AChR-rich membrane extracts stained for CK activity and directly compared with extracts containing CK from brain and skeletal muscle of *T. marmorata* (5). Second, isoelectric point and electrophoretic mobility under denaturing conditions of the *T. marmorata* polypeptides in question (M_r 42,000, pI 6.3–6.5) point to B-type CK, because under the same conditions M-CK is known to run with a subunit M_r of 40,000 ahead of actin (28). In addition, immunological evidence—although obtained by the cross-reaction of rabbit anti-chicken CK antibodies with *T. marmorata* CK—demonstrated that, of the two CK types, only anti-B-CK antibodies cross-reacted with the membrane-associated M_r 42,000 *Torpedo* polypeptides, whereas both B and M antigens were found in total electric organ (5). The present immunohistochemical study clarifies this point even further,



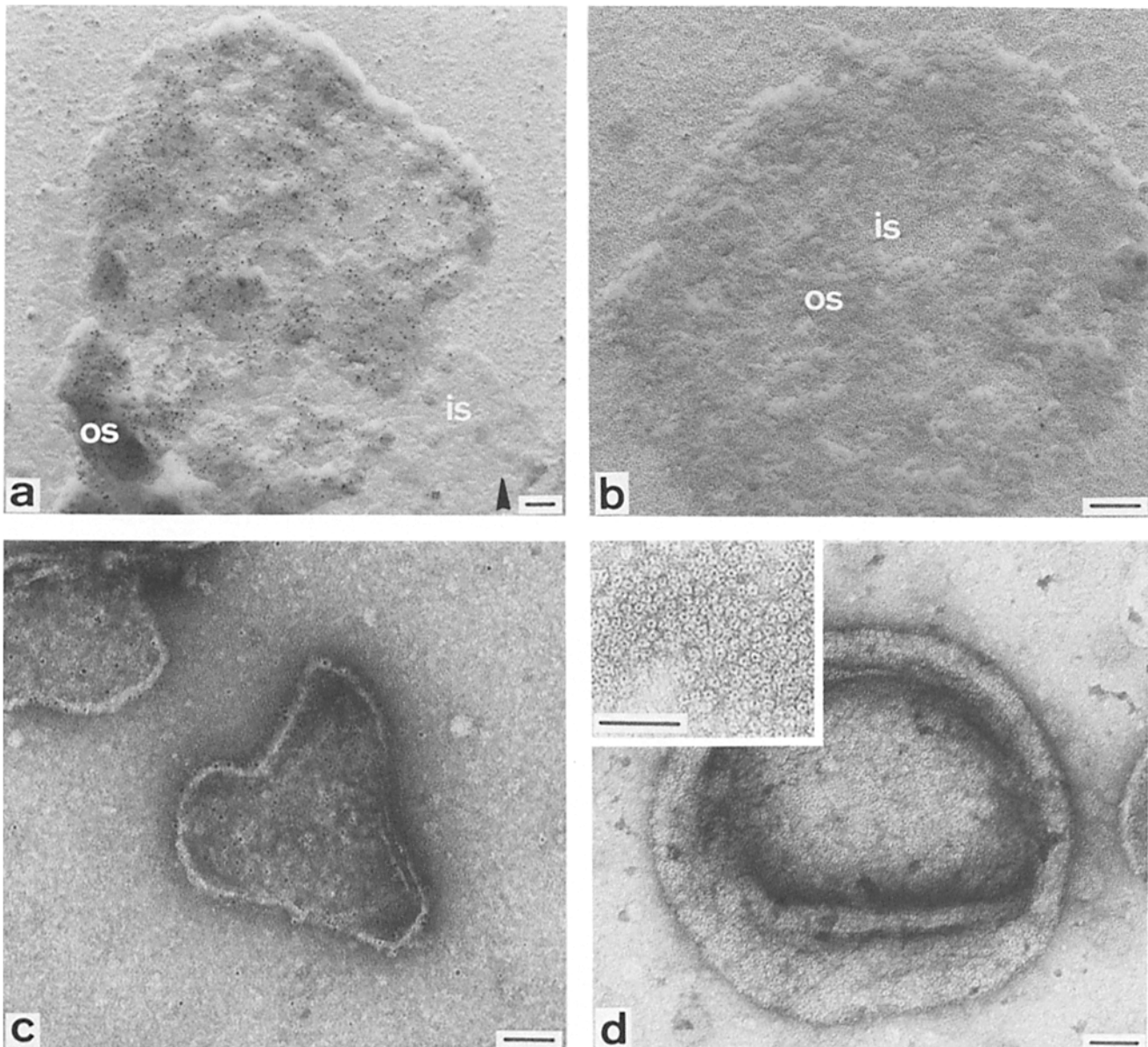
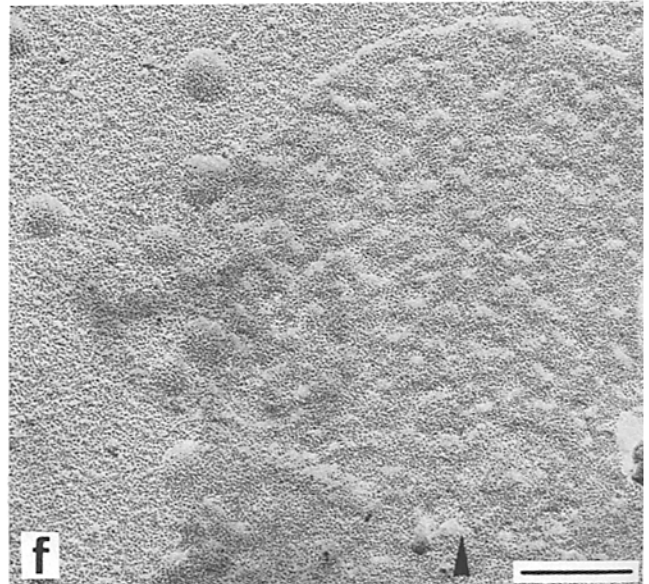
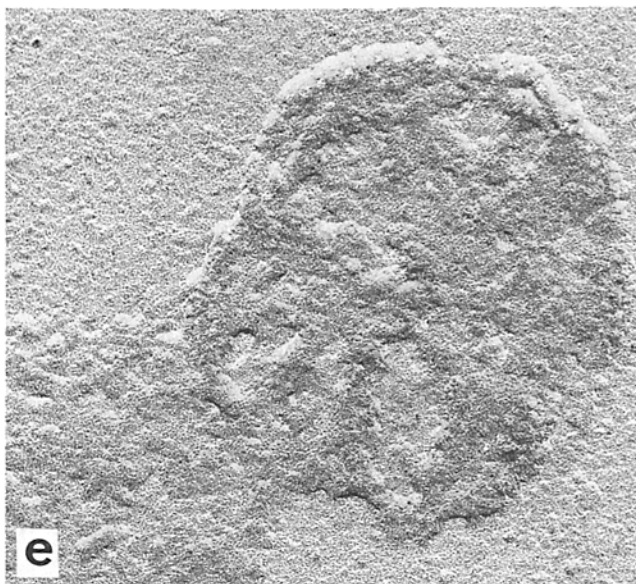
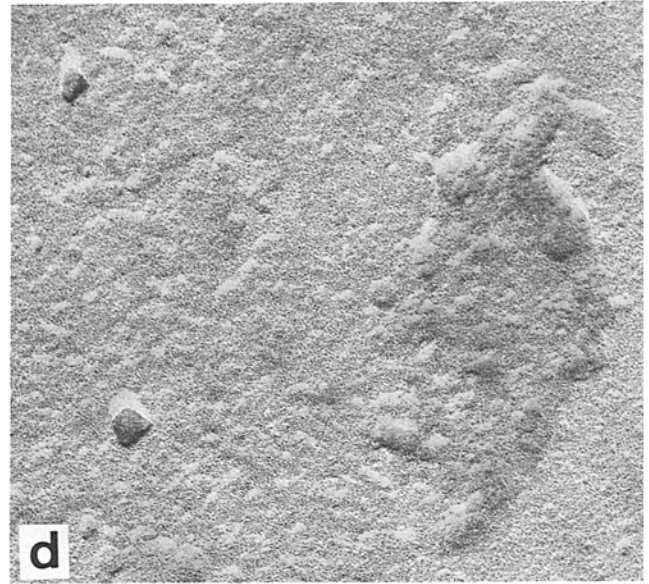
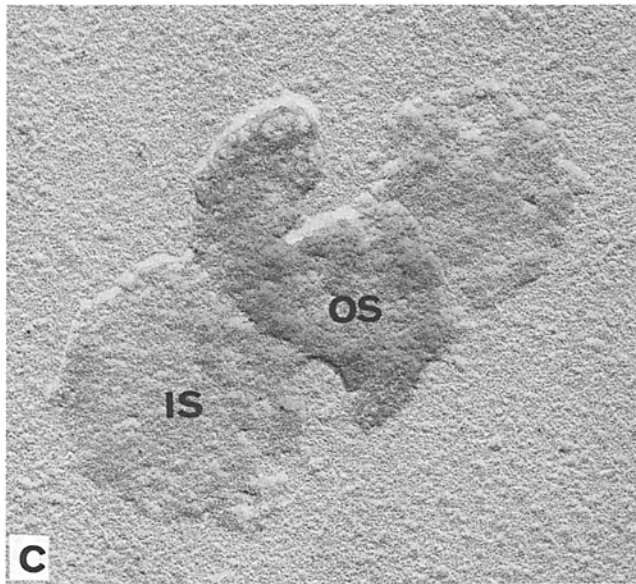
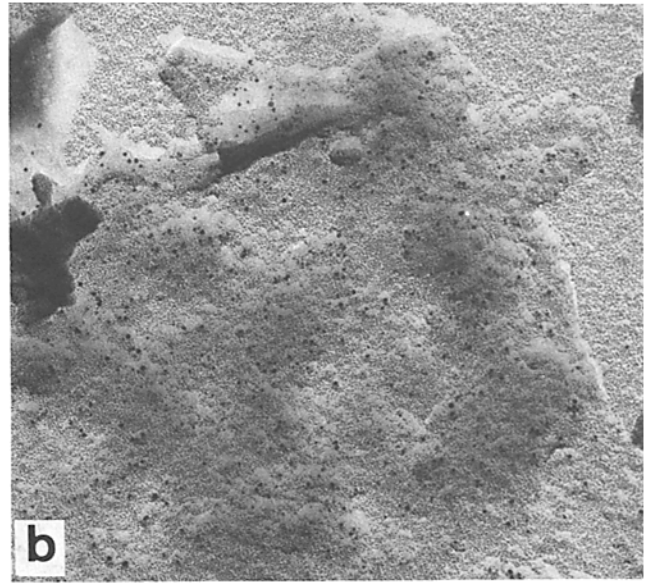
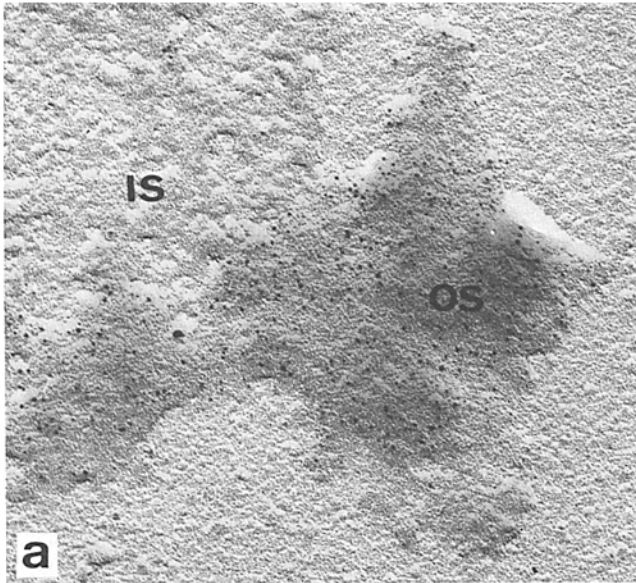


FIGURE 4 Immunohistochemical staining of isolated AChR-rich membrane vesicles with anti-B-CK antibodies and protein A-gold. (a) AChR-rich membranes adsorbed onto carbon-coated grids were incubated with 2 $\mu\text{g}/\text{ml}$ of rabbit anti-chicken B-CK antibody followed by 1:100 diluted protein A-gold solution. Specimens were subsequently freeze-dried and unidirectionally shadowed with Ta/W. Note the almost exclusive labeling of the presumptive outer surface (os) of the fenestrated vesicle. Patches of the inner surface (is) of the vesicles (one step down after shadowing) are free of protein A-gold. Direction of shadowing is indicated by the arrowhead. $\times 150,000$. (b) Same after incubation with identical concentrations of control IgG and protein A-gold. Note the absence of significant labeling. $\times 150,000$. (c) AChR-rich membranes after incubation with 2 $\mu\text{g}/\text{ml}$ of anti-B-CK antibody followed by 1:100 diluted protein A-gold and negative contrasting with 1% aqueous uranyl acetate. Note the labeling of the outer side of the vesicle with anti-B-CK antibodies. $\times 150,000$. (d) Same but after direct negative staining without incubation with antibody or protein A-gold. Note the presence of AChR particles typical of these postsynaptic receptor-rich membranes. (Inset, $\times 200,000$) Bars, 100 nm.

FIGURE 3 Immunohistochemical localization of CK in ultrathin sections of *T. marmorata* electrocytes. (a) Ultrathin cryosection through the dorsal noninnervated face (nif) of a *Torpedo* electrocyte after immunolabeling with 2 $\mu\text{g}/\text{ml}$ of specific rabbit anti-chicken B-CK IgG followed by 1:100 diluted GarG. Note the association of B-CK with the dorsal membrane sac system typical of the noninnervated face. $\times 57,000$. (b) Corresponding control section through a similar region of the noninnervated face (nif) after incubation with 10 $\mu\text{g}/\text{ml}$ of control IgG followed by 1:100 diluted GarG. Note the absence of significant labeling in the control. $\times 75,000$. (c) Ultrathin cryosection through the ventral innervated face (if) of a *T. marmorata* electrocyte after immunolabeling with 2 $\mu\text{g}/\text{ml}$ of anti-B-CK IgG followed by 1:100 diluted GarG. Note the heavy labeling of the postsynaptic membrane (psm) with its typical invaginations towards the cytoplasm (cp) of the electrocyte indicating an association of B-CK with the postsynaptic membrane. Labeling of the synaptic vesicles in the presynaptic region (n) is also observed. $\times 120,000$. (d) Corresponding control section through a similar region of the innervated face (if) of an electrocyte showing no significant labeling of the postsynaptic membrane (psm) after incubation with 2 $\mu\text{g}/\text{ml}$ of control IgG followed by 1:100 diluted GarG. $\times 75,000$. Bars, 200 nm.



dispelling the previously discussed possibility of an adventitious binding of B-CK to the postsynaptic membrane (4). A specific association of B-CK and not M-CK, with the postsynaptic membrane is supported by the specific labeling with anti-B-CK antibodies in situ observed in cryosections. B-CK seems to be strongly associated with the AChR-rich membranes, resisting extraction by extensive washing at physiological ionic strength and pH and, after mild prefixation with formaldehyde, even resisting extraction by SDS and Triton X-100. The membrane-associated CK, however, can be extracted by alkali treatment (4) together with the other ν -proteins, one of which has been attributed to actin (19) and another to a protein kinase (16).

The sidedness of the B-CK with respect to the postsynaptic membrane could not be determined unambiguously by immunolabeling of intact electrocytes in ultrathin sections; gold particles could be seen on both sides of the rather thick postsynaptic membrane complex (Fig. 3c). On the other hand, immunohistochemical labeling of freeze-etched, isolated AChR-rich vesicles showed a clear one-sided labeling. AChR membranes are thought to be predominantly right-side-out, sealed vesicles (2) and are presumably adsorbed as such on carbon films rendered hydrophilic by glow discharge (22). Furthermore, on average, >90% of the AChR particles in these vesicles are exposed right-side-out (46). Vesicles often break open, especially after freeze-drying, exposing simultaneously patches of single layers of the membrane and intact double membrane layers. This gives rise to a fenestrated appearance (Fig. 4a). It is in such vesicles that the gold particles appear to be circumscribed to the double-layered surfaces. However, vesicles deposited on hydrophilic supports and submitted to uranyl acetate contrast also break open frequently, exposing large areas of the inner, "cytoplasmic" face of the rightside-out vesicles (1, 46). Immunogold particles were also seen throughout the surface of these vesicles (Fig. 4c). Other studies aimed at the subcellular localization of the so-called ν -proteins have also faced similar difficulties. Whereas a variety of morphologic, immunocytochemical, and biochemical studies indicate that some of the ν -proteins are located on the inner, cytoplasmic face of the AChR-rich membranes (1, 12, 31, 33, 44), other studies indicate the presence of ν -proteins on both membrane faces (13). The heterogeneity of the ν -proteins (18) and the lack of selective probes for their constituents, however, did not permit a straightforward solution to this controversy. Monoclonal antibodies against the more basic ν_1 -proteins have only recently become available, enabling the localization of this species on the inner face of the postsynaptic membrane (26). The localization of the intermediate pI species (the ν_2 -proteins), which we have identified as B-CK in AChR-rich vesicles (4, 5), could have escaped detection when rather crude antigens were used (31) because of their minor contribution to the alkali extract of AChR membranes (4, 18). The sidedness of B-CK in the postsynaptic membranes is therefore a still open question.

Immunocytochemical experiments that use both anti-B-CK and anti- ν_1 -protein (26) are currently under way in order to clarify this issue.

The direct localization of B-CK at the synaptic vesicles (Fig. 3, *inset*) confirms biochemical evidence of an association of CK activity with brain preparations enriched in this subcellular structure (14). It is likely that the bound CK is functionally coupled with the ATP-dependent energy consumption in transmitter release as an ATP-regenerating system (7). A functional coupling of the B-CK found at the dorsal membrane-sac system with the Na/K-ATPase may be postulated. CK bound to the postsynaptic membrane complex may be involved either in receptor function by being coupled to ATP in the use of processes such as internalization of the receptor, or by working in tandem with the postsynaptic protein kinase that has recently been identified as a component of the AChR-rich membranes (16). Specific association of CK—a mostly soluble enzyme in muscle—with the inner mitochondrial membrane, the sarcoplasmic reticulum, and the myofibrillar apparatus, has been demonstrated. Functional coupling of CK with the ATP/ADP translocase, the Na/K ATPase, and the Ca^{2+} ATPase, respectively, has been postulated (for review see reference 39). As in the case of muscle (40), the CK isoenzymes of *T. marmorata* seem to exhibit isoenzyme-specific segregation with differential, compartmentalized locations. Thus, the physiological significance of the fact that different isoenzymes may be present within one cell may lie in the ability of the different CK isoenzymes to interact selectively with different cellular structures and their constituents.

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Note Added in Proof: The initial finding of Barrantes et al. (references 4 and 5) that CK is closely associated with the AChR-rich membrane and that CK is identical to the 43,000-D ν_2 -protein species of isolated AChR-rich vesicles (Barrantes, F., A. Braceras, G. Mieskes, E. C. Toren, H. A. Caldironi, M. Roque, and A. Zechel, 1985, *J. Biol. Chem.* 260:3024–3034) has been confirmed by Gysin et al. (19) and by Giraudat et al., (Giraudat, J., A. Devillers-Thiery, J.-C. Perriard, and J. P. Changeux, 1984, *Proc. Natl. Acad. Sci. USA.*, 81:7313–7317), respectively. However, there remains a discrepancy

FIGURE 5 Localization of B-CK on purified AChR vesicles opened by the jet-stream squirting technique (9, 25) after adsorption onto carbon-coated grids and incubation with 2 $\mu\text{g}/\text{ml}$ of anti-B-CK IgG (a and b); 5 $\mu\text{g}/\text{ml}$ of control IgG (c and d); or PBS alone (e and f). All samples were subsequently incubated with 1:100 diluted protein A-gold, washed, freeze-dried, and unidirectionally shadowed with Pt/C. AChR vesicles shown in a, c, and e were mildly fixed on the grid with 3% formaldehyde and 0.1% glutaraldehyde after being submitted to the jet stream and further treated with 0.1% SDS and 0.1% Triton-X-100 before immunolabeling. *os*, presumptive outer face of AChR-rich membranes; *is*, inner presumptive face of vesicles. Arrowhead indicates direction of shadowing. Bar, 100 nm. $\times 150,000$.

as to whether ν_2 -protein represents B- or M-type CK. The results presented here are based on immunological evidence using rabbit anti-chicken B- and M-CK antibodies which show interspecies cross-reactivity with *T. marmorata* CKs. In our hands the ν_2 -protein spots on two-dimensional gels of purified AchR-rich membranes cross-react only with anti-chicken B-CK antibodies and not with anti-M-CK antibodies (4, 5). Therefore, ν_2 -protein has been interpreted to be B-type CK (5). In addition, the lack of cross-reactivity on immunoblots of AchR-rich membranes with anti-chicken M-CK antibodies has recently been confirmed also by immunogold labeling of AchR-rich membrane vesicles (D. Walzthöny, unpublished observation). Thus, even though our immunoblotting analysis and immunogold labeling are in agreement (references 4 and 5, and Barrantes, F., A. Braceras, G. Mieskes, E. C. Toren, H. A. Caldironi, M. Roque, and A. Zechel, 1985, *J. Biol. Chem.* 260:3024–3034) the interpretation of CK isoenzymes in *T. marmorata* may not be as simple as in the homologous chicken system, for *Torpedo*, like other fish, may have multiple forms of M- and/or B-type CK isoenzymes which may show different cross-reactivities with our subunit-specific anti-chicken CK antibodies, especially after denaturation of the antigens. Also, recently it became clear that there is an unexpectedly high amino acid sequence homology between M- and B-type CKs and that cross-hybridization of rabbit M- and B-CK cDNAs can be observed (Putney, S. et al., 1984, *J. Biol. Chem.* 259:14317–14320).

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