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Creatine kinase activity in the *Torpedo* electrocyte and in the nonreceptor, peripheral ν proteins from acetylcholine receptor-rich membranes

(nicotinic cholinergic receptor/membrane-associated enzyme/synapse/peripheral membrane proteins/muscle dystrophies)

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ABSTRACT **The nonreceptor, peripheral ν proteins (M_r 43,000 proteins) are conspicuous components of the acetylcholine receptor-rich membranes and the *Torpedo* electrocyte, so far devoid of any known enzymatic function. Creatine kinase (adenosine 5′-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2) is identified in distinct polypeptides belonging to the family of ν proteins. Embryonic (70- to 90-mm embryos), neonatal, and adult electric organs of *Torpedo marmorata* contain two isoenzymes of creatine kinase: the BB (brain) and the MM (muscle) forms. The proportion of the two isoenzymes does not appear to change in the course of ontogenic and postnatal development. Only the BB isoenzyme appears to be associated with the acetylcholine-rich membranes in adult *Torpedo*. The creatine kinase can be purified to homogeneity by chromatographic procedures that exploit the richness in free sulfhydryl groups of the enzyme. Specific activities of 150 units/mg are obtained from electric tissue. The enzyme subunits identified by two-dimensional gel electrophoresis and immunoblotting techniques have pI values in the 6.0–6.5 region and apparent molecular weights in the 40,000–43,000 range, the latter values depending on redox conditions.**

A group of proteins named the 43K-protein (1), the 43,000 M_r protein (2), the ν-peptide (3), the ν-doublet (4), or the 43,000-dalton proteins (5) appears to be one of the predominant components of the electrocyte and the second most abundant constituent of acetylcholine receptor (AcChoR)-rich membrane fragments obtained therefrom. These proteins are also found in the synaptic region of mammalian muscle (6). Initially believed to be associated with the site of action of noncompetitive antagonists, this group of proteins (hereafter referred to as ν proteins) was left in a state of "functional orphanage" after it was demonstrated that their extraction was without effect on the binding of local anesthetics and on the AcChoR-controlled ion permeability (2, 7). It was later shown that depletion of the ν proteins gives rise to enhanced rotational (8–10) and translational (11) motion of the AcChoR and to modifications of the receptor packing habit (4). In the latter study it was proposed that the missing proteins play a role in processes such as synapse formation during ontogenesis, receptor clustering, and stabilization of the adult synapse. The ν proteins also appear to confer stability on the AcChoR and prevent it from thermal denaturation (12) and proteolysis (13–15).

The present work describes the identification of ν protein fractions from *Torpedo* electric tissue and AcChoR-rich membrane fragments as creatine kinase (adenosine-5-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2). The enzyme is one of the kinases that catalyze phosphate group transfer to a guanidino group. Specifically, it catalyzes the reaction

$$MgADP^- + \text{phosphocreatine} + H^+ \rightleftharpoons MgATP^{2-} + \text{creatine}.$$

The enzyme is responsible for the maintenance of ATP levels in muscle. This and other tissue having high energy demands (brain, heart) have the highest concentrations of creatine kinase (16) and phosphocreatine (17). The electrocyte of electric fish is also a cell with high energy expenditure and embryological similarities with muscle. In fact, the developing electroblast in the electric tissue primordia has myoblast-like characteristics, even displaying spontaneous contractile activity (18). The presence of creatine kinase in the adult electrocyte might thus represent an activity preserved through evolution, whose relationship with tissues possessing nicotinic (electric tissue, skeletal muscle, brain) and muscarinic (brain, heart) AcChoRs remains to be elucidated.

MATERIALS AND METHODS

Purification Procedures. AcChoR-rich membranes from *Torpedo marmorata* were isolated by the procedures previously reported (19). Alkaline extraction of nonreceptor proteins was done essentially as described in ref. 2 with the modifications given in ref. 20.

Total *Torpedo* electric tissue or dorsal muscle homogenates, or the above extracts of purified AcChoR membranes, were submitted to successive extractions with 50% and 70% (vol/vol) ethanol as described (21) for the purification of muscle creatine kinase. The partially purified enzyme was then dialyzed against the appropriate buffer and simultaneously concentrated under mild negative pressure by using Schleicher & Schüll dialysis bags in the presence of 1–2 mM 2-mercaptoethanol and chromatographed through *p*-hydroxymercuribenzoate-Sepharose CL-6B (22).

Analytical Procedures. Separation and identification of the native creatine kinase isoenzyme by paper electrophoresis was carried out on Gelman Sepraphore III strips equilibrated with 60 mM sodium barbital buffer, pH 8.6, containing 0.06% 2-mercaptoethanol. The total homogenates of *Torpedo* brain, dorsal muscle, or electric tissue were electrophoresed at 4°C for 150 min at 250 V. Creatine kinase was identified with a specific chromogenic coupled enzyme reaction (23). $NaDodSO_4$ gel electrophoresis was done according to Laemmli (24). The two-dimensional system of O'Farrell (25) was used when isoelectric focusing preceded electrophoresis in the second dimension. Thiol

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Abbreviations: AcChoR, acetylcholine receptor; B and M, subunits of brain and muscle type creatine kinase.

modifications of the membranes were effected as reported in ref. 26. Immunoblotting on nitrocellulose paper was carried out as described (20), using rabbit antisera against chicken BB (brain) or MM (muscle) types of creatine kinase. The immune replicas were first incubated for 2 hr in 5% horse serum in 50 mM Tris·HCl buffer, pH 8.0, followed by 12-hr incubation in the same buffer containing either the MM or the BB antiserum at a 1:100 dilution. The second antibody was goat anti-rabbit IgG conjugated with fluorescein isothiocyanate and was also used at a 1:100 dilution. Enzyme activity was assayed by following the recommended "optimized procedure" of the German Society for Clinical Chemistry (see ref. 27). Protein determination was done according to Lowry *et al.* (28) except for samples containing thiol reagents, in which case the Bio-Rad procedure was employed. In both cases the standard was bovine serum albumin. The toxin binding capacity was measured by the DEAE-cellulose filter assay (29).

RESULTS

Fig. 1 shows a cellulose acetate paper electropherogram of total homogenates from *Torpedo* dorsal muscle, electric tissue, brain, and AcChoR membranes, developed under conditions specific for creatine kinase. *Torpedo* electric tissue (Fig. 1, lane *c*) contains predominantly MM creatine kinase, an intermediate species (presumably the hybrid MB form), and much smaller amounts of BB creatine kinase. The latter has the same electrophoretic mobility as the main species in *Torpedo* brain, which also shows a second component with the mobility of chicken mitochondrial creatine kinase. *Torpedo* dorsal muscle (Fig. 1, lane b) shows, in addition to the main MM species, the hybrid MB form and a faint myokinase band in between. AcChoR membranes have only a very faint band at the level of BB-creatine kinase (Fig. 1, lane d, arrow).

Immunoblotting techniques are 2–3 orders of magnitude more sensitive than the color-developing coupled enzyme reaction on cellulose acetate strips. A single band is made apparent in total extracts of adult electric tissue, separated by polyacrylamide gel electrophoresis under denaturing conditions, upon binding of chicken antibodies against either the brain (BB) (Fig. 2, lane e′) or the muscle (MM) (Fig. 2, lane e) type of creatine kinase antigen. The strength of the antibody–antigen reaction is not a measure, however, of the amount of antigens present. The stronger fluorescence with anti-BB creatine kinase is due

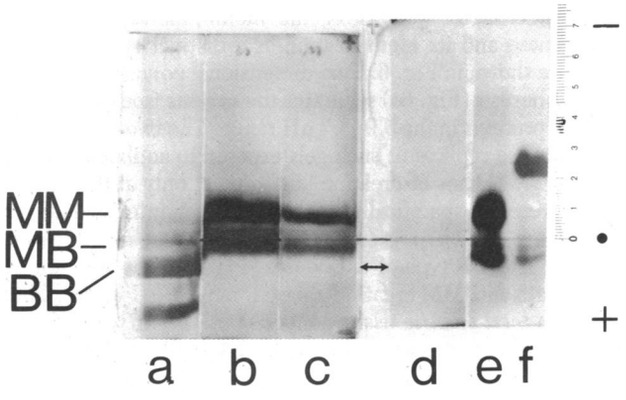

FIG. 1. Cellulose polyacetate paper electropherogram of total homogenates from *Torpedo* brain (lane a), dorsal muscle (lane b), electric tissue (lane c), and AcChoR-rich membranes (lane d) overlaid with creatine kinase substrates in coupled enzyme reaction (23). Standards are chicken MM (lane e) and BB (lane f) creatine kinase. The origin is indicated by the horizontal line and the ● symbol. The double arrow shows the position of the faint BB creatine kinase bands in electric tissue and AcChoR-rich membranes.

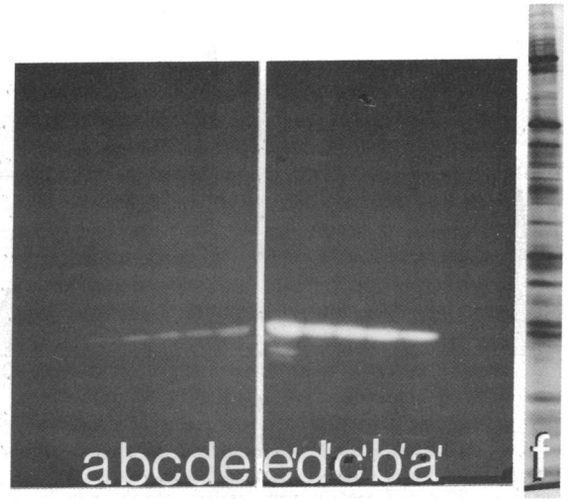

FIG. 2. Creatine kinase through ontogenesis of electric tissue and identification of creatine kinase species by immunoblotting. Nitrocellulose paper replicas of a slab gel of total homogenates from electric organs of 70-mm (a, a′), 80-mm (b, b′), and 90-mm (c, c′) *T. marmorata* embryos and neonatal (d, d′) and adult (e, e′) electric tissue incubated with rabbit antibodies to MM (chicken muscle) creatine kinase and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (a–e). Lanes a′–e′ are the corresponding anti-BB immune replicas processed in parallel in an identical manner. Sixty-microgram samples of protein were applied to each lane. Lane f is a Coomassie blue-stained polyacrylamide gel electropherogram of the 90-mm *Torpedo* embryonic tissue extract.

to the closer antigenic similarity between *Torpedo* and chicken BB creatine kinase in comparison to MM creatine kinase (unpublished observations). Embryonic electric tissue (70-, 80-, and 90-mm embryos) also contains both isoenzymes (Fig. 2, lanes a–c and a′–c′). Electric organ of neonatal *Torpedo* follows the same pattern (Fig. 2, lanes d and d′). Both isoenzymes are thus preserved throughout ontogenetic development without apparent quantitative variations in their proportion.

Exploitation of endogenous chemical groups in the *ν* proteins was suggested by their solubility properties, dependent on the presence of thiol reagents (1, 30), and their ability to be heavily labeled with iodoacetamide (30) and *N*-ethylmaleimide (3). Furthermore, organomercurial-agarose media have been successfully employed for the purification of creatine kinase from human skeletal muscle, also exploiting the richness in thiol groups of this enzyme (22). Fractions having specific activities of 270 units/mg were obtained from ethanol extracts of *Torpedo* dorsal muscle at a yield of 50–70% by using Sepharose CL-6B derivatized with *p*-hydroxymercuribenzoate. The ethanol extracts from adult electric tissue submitted to chromatography in this medium yielded a similar elution profile (Fig. 3). The bulk of the creatine kinase activity and protein eluted with a pulse of *p*-hydroxymercuribenzoate. Subsequent application of a 2–100 mM 2-mercaptoethanol gradient resulted in further elution of creatine kinase activity.

Fig. 4 shows the $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of the fractions obtained by thiol-Sepharose chromatography of electric tissue extracts. The creatine kinase-rich fractions II and III are electrophoretically homogeneous, with apparent M_r 40,000–43,000 (Fig. 4, lanes f and g), and migrate below *Torpedo* actin (Fig. 4, lane e), which elutes with the column void volume. The creatine kinase activity is therefore present in a protein having an apparent molecular weight equivalent to that of the *ν* proteins of AcChoR membranes (4, 20). This is further shown in Fig. 5, where the thiol-dependent variations in creatine kinase electrophoretic mobility are made

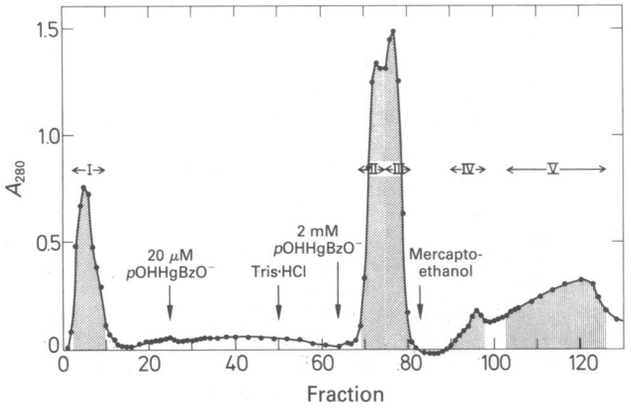

FIG. 3. Purification of creatine kinase from the electric tissue of *T. marmorata*. Samples of ethanol extracts of total electric tissue containing about 20 mg of protein were loaded onto the *p*-hydroxymercuribenzoate-Sepharose column (1.8 ml) equilibrated with 50 mM Tris·HCl buffer, pH 7.4. After elution of nonadsorbed protein, pulses of *p*-hydroxymercuribenzoate (*p*OHHgBzO⁻) at increasing concentrations were applied. The main creatine kinase activity was eluted with 2 mM reagent in coincidence with the main protein band (peaks II and III, specific activity 75 and 68 units/mg of the total activity recovered). The total recovery of these columns was 15–20% (activity) and 27–35% (protein). Maximal specific activity was recovered in the fraction eluting with a 2–100 mM 2-mercaptoethanol gradient (IV, 157 units/mg). The corresponding polypeptide patterns are shown in Fig. 4.

apparent. In the absence of reducing or alkylating reagents, the enzyme displays two main bands of almost equal Coomassie blue intensity in $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 5, lane b). One of these bands runs with a mobility

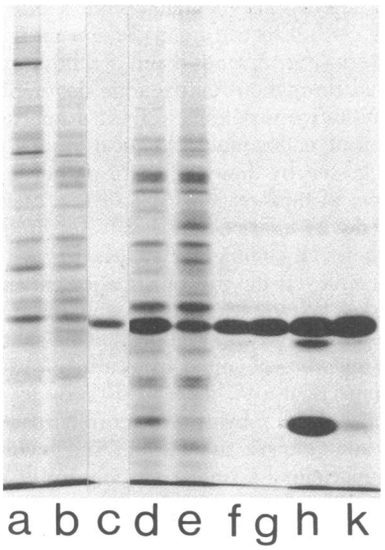

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of electric tissue creatine kinase fractions obtained by thiol-Sepharose chromatography (Fig. 3). The starting material, the total homogenate of electric tissue of *T. marmorata*, is shown in lane a. Lane b corresponds to the product of the 50% ethanol extraction from this homogenate. Lane c shows rabbit muscle creatine kinase. Lane d is the material applied to the thiol column—i.e., the product of the combined ethanol extractions. Lanes e–k correspond to peaks I, II, III, IV, and V eluted from the thiol column as shown in Fig. 3, having specific activities of 25, 75, 68, 157, and 112 units/mg, respectively. The lower specific activity in the electrophoretically pure fractions II and III is most likely due to the residual inhibitory effect of *p*-hydroxymercuribenzoate. As shown by Ennor and Rosenberg (31), inhibition by this ligand is only partly reversible.

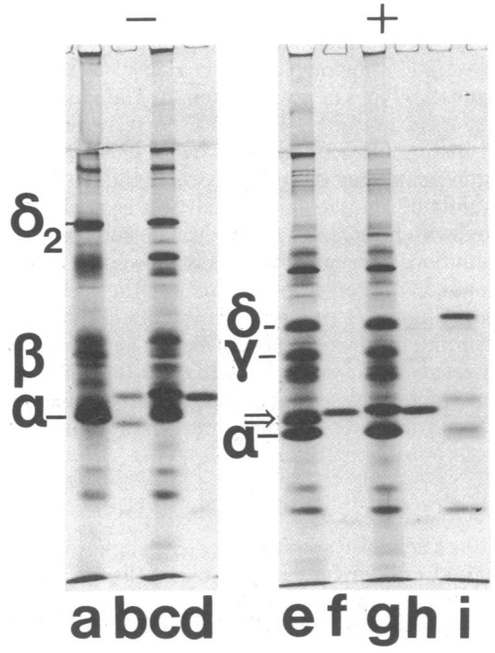

FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of AcChoR-rich membranes (lanes a, c, e, and g) and rabbit muscle creatine kinase (lanes b, d, f, and h) under nonreducing (−) and reducing (+) conditions (10 mM dithiothreitol, 30 min, 20°C) with (lanes c, d, g, and h) or without (lanes a, b, e, and f) subsequent alkylation with *N*-ethylmaleimide (10 mM, 30 min, 20°C). Lanes b, d, f, and h contain 1.5 μg of creatine kinase. Two main bands are observed under nonreducing conditions in the absence of alkylating agents (b). Only one main band results from alkylation (d) or reduction not followed (f) or followed (h) by alkylation. Lane i contains standards; from top to bottom: bovine serum albumin (M_r, 68,000), ovalbumin (M_r, 45,000), aldolase (M_r, 40,000), and chymotrypsinogen (M_r, 25,000). The double arrow points to the position of the ν protein doublet (4). α, β, γ, and δ are subunits of AcChoR.

slightly higher than that of the α subunit of the AcChoR and the other has the same mobility as the slow component of the ν proteins, resembling the pattern reported by Williams *et al.* (32) of two polypeptides of M_r 42,100 and 40,300. Reduction or alkylation simplify the pattern to a single band (Fig. 5) both in the case of muscle creatine kinase and in the case of the ν proteins.

The microheterogeneity of the membrane-associated creatine kinase and its identification as a distinct type of isoenzyme are shown in Fig. 6. Two-dimensional polyacrylamide gel electrophoresis (Fig. 6*a*) separates the various isoelectric forms of the ν proteins in the 5.9–8.7 pH range. The two-dimensional nitrocellulose replicas of such gels exposed to antibodies against BB and MM forms of creatine kinase react only at the level of the ν proteins, specifically in the region of intermediate pH (≈6.5). Two major spots and a minor one crossreact exclusively with the anti-BB type of antibodies (Fig. 6*b*); the immune reaction with anti-MM creatine kinase is negative (Fig. 6*c*). Thus, although *total* electric tissue contains both isoenzymes (Fig. 2) *only the BB form is associated with AcChoR membranes.*

DISCUSSION

Creatine kinase activity has been found in the electric tissue of *T. marmorata* and in the AcChoR-rich membranes. Purification of this activity is accompanied by the enrichment of the fractions in polypeptides having electrophoretic properties like those of trout creatine kinase isoenzymes (33) and the ν proteins found in AcChoR membranes (1–5, 7, 9, 12–15) and mam-

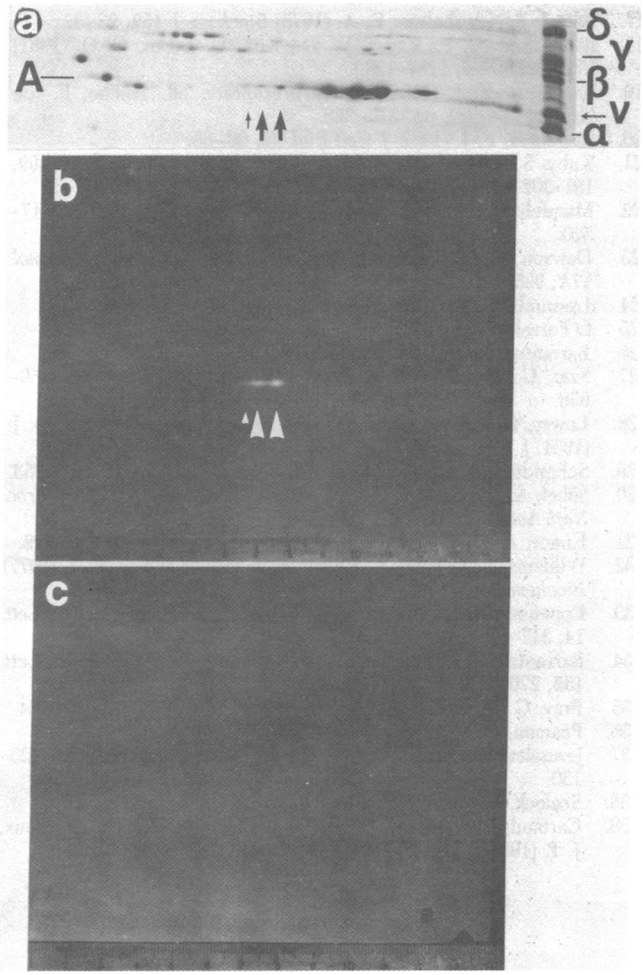

FIG. 6. Two-dimensional polyacrylamide gel electrophoresis (*a*) and corresponding immunoblots (*b* and *c*) of AcChoR membrane polypeptides. The nitrocellulose paper replicas were incubated as in Fig. 2 with antibodies to BB creatine kinase (*b*) and MM creatine kinase (*c*). Two main spots and a minor one (arrows) give a positive reaction with the antibodies to BB (*b*) but not with anti-MM creatine kinase (*c*), at the level of the ν protein subspecies of intermediate pI value (pI 6.0–6.5, M_r 40,000–43,000, see arrows). A, actin; α, β, γ, and δ are subunits of AcChoR.

malian muscle (6). The ν proteins are membrane-associated proteins, their antigenic determinants being mainly exposed on the cytoplasmic face of the membrane (20). Their degree of association with the membrane is different for the three groups of ν proteins defined by their isoelectric points (5). Gysin *et al.* (5) concluded that the more basic (pI 7.2–7.8) of these proteins were more tightly bound, because they copurified with the AcChoR membranes obtained by affinity partitioning, whereas the more acidic species (pI 6.0–7.2) were more loosely bound to the AcChoR membranes prepared by the above technique. They did not show whether such acidic species copurified with the membranes prepared by sucrose gradients as in the present case. The acidic species are also found in substantial amounts in the soluble fraction of electric tissue homogenates.

Here we show that within the group of ν proteins, the subspecies of intermediate pI value (6.0–6.5) correspond to the BB creatine kinase. The enzyme can be purified to homogeneity from total electric tissue or from the AcChoR membranes themselves (34). Within the sensitivity of the techniques employed only the BB isoenzyme is present as a ν protein in the AcChoR membranes, whereas both BB and MM creatine kinase are present in total electric tissue. Because homomeric MM creatine kinase synthesis in myogenic cultures is preceded by the simultaneous biosynthesis of the two isozymes, preservation of the BB form as a membrane-associated species might have some functional significance. The kinetic properties of the soluble and membrane-associated creatine kinase do not differ (34), suggesting either that the association with the membrane is trivial or that it obeys rules other than enzymatic facilitation via coupling with the membrane structure. Such structure is densely packed with AcChoR molecules, and the obvious implication is that an enzyme related to the energy metabolism is involved in a process subserving AcChoR metabolism itself. One such process is the internalization of the AcChoR prior to its catabolic destruction. The internalization is an energy-consuming step mediated by endocytosis. The neighborhood of AcChoR and ν proteins revealed by cytochemical techniques (6) could thus reflect a topographically necessary relationship with the structure where the process is triggered.

Another aspect of the ν protein–AcChoR relationship in which creatine kinase might be involved concerns the rotational and translational mobility of the AcChoR molecule. The AcChoR in membranes depleted of the ν proteins display a fast rotational correlation time characteristic of the 9S, M_r 250,000 monomeric species (10), which is conspicuously absent from alkylated membranes, predominantly made up of the 13S dimer (20). Alkylation of AcChoR membranes also abolishes creatine kinase activity, due to the presence of essential thiols in the active site of this enzyme (31). There also appears to be no hindrance to the translational motion of the monomers and dimers in ν-protein-depleted membranes (11). The participation of the ν proteins in the thiol-dependent equilibria of the AcChoR has been proposed (19, 20), and emphasis has been put on the difference between the state of the membrane *in vitro* and that existent in the metabolically active living synapse (26). Whether the finding of a thiol-dependent enzymatic function related to the energy metabolism in the ν proteins bears relationship to the corresponding thiol-dependent affinity states (26) and oligomeric equilibria (19, 20) of the AcChoR remains to be established.

Finally, a hypothesis relating the putative coexistence of creatine kinase and AcChoR in the synaptic region merits discussion. One of the pathognomonic signs in various muscle myopathies is the increased serum levels of creatine kinase. In Duchenne muscle dystrophy this symptom parallels a reduced creatine kinase activity in the muscle (35) and a leak of creatine kinase from this tissue which appears more selective than in other myopathies (36). Interestingly, it is in this dystrophy that a consistent abnormality of the synapse restricted to the postsynaptic membrane has been described (37). Such pathology is not associated with degeneracy of the nerve terminals, but the postsynaptic folds are shallower than normal (referred to as "focal atrophy"; ref. 37). The ν proteins appear to be localized at the postsynaptic membrane in skeletal muscle (6), more specifically facing the cytoplasmic face of the membrane in *Torpedo* (20). Furthermore, postsynaptic densities disappear upon alkali stripping of AcChoR membranes (38, 39). Could it be that (*i*) a correlation exists between the ultrastructural alterations found in Duchenne muscular dystrophy (37) and depletion of ν proteins from the synapse and (*ii*) a primary defect in the fraction of membrane-associated or cytoplasmic ν proteins underlies this X-linked recessive disease or other muscle myopathies showing creatine kinase depletion? The tools to test these hypotheses are currently at hand.

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