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Biochemistry

ν_1, a M_r 43,000 component of postsynaptic membranes, is a protein kinase

(acetylcholine receptor/phosphorylation)

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ABSTRACT **Acetylcholine receptor-enriched membranes from the electric organ of *Torpedo californica* show a major band at M_r 43,000 on NaDodSO$_4$/polyacrylamide gels. This band is composed of three polypeptides: ν_1, ν_2, and ν_3. Polypeptide ν_1 has been found to be localized exclusively at the innervated face of the electrocyte and at the neuromuscular junction in rat muscle. We show here that monoclonal antibody to ν_1 precipitates a radioactive M_r 43,000 polypeptide from detergent-solubilized extracts of *Torpedo* membranes covalently labeled with periodate-oxidized [α-^{32}P]ATP. The monoclonal antibody also precipitates protein kinase activity from neutralized pH 11 extracts of the acetylcholine receptor-rich membranes. These data suggest that ν_1 is a postsynaptic membrane protein kinase.**

Acetylcholine receptor (AcChoR) phosphorylation in receptor-enriched membranes from *Torpedo californica* has been demonstrated in several laboratories (1–5). However, the protein kinase(s) that phosphorylates the receptor has not been identified. Our laboratory has shown that an alkali-extractable M_r 43,000 polypeptide(s) associated with AcChoR-enriched membranes binds ATP and phosphorylates casein (1). AcChoR-enriched membranes have been found to contain three alkali-extractable polypeptides of M_r 43,000, ν_1, ν_2, and ν_3, which can be separated by two-dimensional electrophoresis (6). ν_2 (pI 6–6.5) and ν_3 (pI 5.6) have been identified as creatine kinase (7, 8) and actin (8, 9), respectively, both of which have ATP binding sites. ν_2 has been reported to be associated with AcChoR-enriched membrane vesicles in addition to being present at high concentrations in the cytosol (6); highly purified membrane preparations, however, appear to have little ν_2 (10). In contrast, ν_1 is only membrane-bound (pI 7.0–7.4) and copurifies with the AcChoR (6, 8, 9). We have used an anti-ν_1 monoclonal antibody (mAb) to demonstrate that ν_1 is a protein kinase.

MATERIALS AND METHODS

AcChoR-Enriched Membranes. Postsynaptic membranes were prepared as described (11). A fraction of these vesicles appear to be permeable to ATP, since phosphorylation of the AcChoR is observed in the absence of detergent (12). Alkali extraction (pH 11) of membranes (10 mg/ml) was carried out for 1 hr at 0°C and the extract was neutralized as described (1). Extracts (≈0.5 mg of protein per ml) were used on the day of preparation.

Preparation of Oxidized [α-^{32}P]ATP. One nanomole of [α-^{32}P]ATP$_{ox}$ was prepared according to a modification (13) of the method of Clertant and Cuzin (14).

Covalent Binding of [α-^{32}P]ATP$_{ox}$ to AcChoR-Enriched Membranes. [α-^{32}P]ATP$_{ox}$ (0.5 μM, 40 μCi; 1 Ci = 37 GBq) was incubated with 20 μg (protein) of AcChoR-enriched membranes in 1.6 mM ouabain/10 mM $MnCl_2$/100 mM NaF/300 mM NaCl/10 mM Tris Cl, pH 7.4/1 mM EDTA containing 5 mM sodium cyanoborohydride (Fluka). The reaction mixture was incubated for 3 hr in the dark at room temperature or as indicated. The reaction was stopped by the addition of sodium dodecyl sulfate, EDTA, and 2-mercaptoethanol as described (1) or by immunoprecipitation.

Immunoprecipitation. The reaction mixture containing [α-^{32}P]ATP$_{ox}$-labeled vesicles (20 μg) was brought to 20 mM EDTA and centrifuged for 10 min in a Beckman Airfuge at 167,000 × *g*. The supernatant was discarded, and the pellet was resuspended in 0.1 M Tris Cl, pH 8/0.1 M NaCl/0.5% Nonidet P-40 (NP-40, Sigma) containing bovine serum albumin (5 mg/ml) and control mouse IgG (2.5 μg/ml). Samples were incubated for 30–60 min at room temperature and then precipitated by a 15-min incubation with *Staphylococcus aureus* cells (Pansorbin, Calbiochem) (which had been prewashed four times in 10 mM sodium phosphate, pH 7.4/0.15 M NaCl/0.25% NP-40) followed by a 10-min centrifugation in a Beckman Microfuge. Eighty microliters of the supernatant from this step was incubated with 10 μl of control (250 μg/ml) or experimental (180 μg/ml) antibody for 1 hr at room temperature and then 16 hr at 4°C.

Samples were then diluted with 0.2 ml of 0.1 M Tris Cl, pH 8/0.1 M NaCl/0.5% NP-40 and incubated for 15 min with 0.02 ml of washed Pansorbin that had been preincubated with unlabeled AcChoR-enriched membranes. Samples were then centrifuged for 2 min in a Beckman Microfuge. The pellets were washed four times with 1 ml of 10 mM sodium phosphate, pH 7.4/0.15 M NaCl/0.25% NP-40/5 mM sodium cyanoborohydride and resuspended in buffer for NaDodSO$_4$/PAGE as described (1).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was carried out by the method of O'Farrell (15) as modified by Porter and Froehner (9).

Immunoblotting. Immunoblotting was carried out according to the method of Towbin *et al.* (16). Proteins in two-dimensional gels were electrophoretically transferred for 2 hr at 100 volts to nitrocellulose paper by use of a Hoefer Transfor cell (TE-42). After incubation and washing in blocking buffer, the replica was incubated for 2 hr with a 1:200 dilution of mAb (180 μg/ml) and then washed four times for 10 min. This was followed by incubation for 1.5 hr with ^{125}I-labeled goat anti-mouse IgG (2 × 10^6 cpm). The paper was then washed, dried, and exposed to Kodak X-Omat film at −70°C with an intensifying screen. After autoradiography, the paper was stained with india ink according to Hancock and Tsang (17).

Phosphorylation of Casein. Neutralized pH 11 extract was immunoprecipitated with control or anti-ν_1 antibody as de-

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Abbreviations: AcChoR, acetylcholine receptor; [α-^{32}P]ATP$_{ox}$, oxidized [α-^{32}P]ATP; mAb, monoclonal antibody; NP-40, Nonidet P-40.

scribed above, but in the absence of bovine serum albumin and NP-40. Samples (20 μl) of the supernatants from the Pansorbin precipitation were assayed for protein kinase activity with casein as substrate. The reaction mixture (0.1 ml) contained 1.6 mM ouabain, 10 mM $MnCl_2$, 100 mM NaF, 240 mM NaCl, 10 mM Tris Cl (pH 7.4), 50 μM [γ-^{32}P]ATP (10 μCi) and 10 μg of casein. After incubation for 30 min at 37°C, the reaction was stopped by addition of EDTA, $NaDodSO_4$, 2-mercaptoethanol and analyzed by electrophoresis according to published procedures (11). Gels were stained, dried, and exposed to Kodak X-Omat film at −70°C with two intensifying screens.

Iodination. Neutralized pH 11 extract (400 μl) was iodinated with 5 mCi of $Na^{125}I$ by lactoperoxidase according to the method of Morrison (18).

mAb. Anti-ν_1 mAb was prepared and characterized by Burden (25). The K_d of the antibody is $\approx 10^{-8}$ M.

RESULTS AND DISCUSSION

The specificity of the mAb was determined by immunoblotting after two-dimensional electrophoresis of AcChoR-enriched membranes (Fig. 1). The mAb reacts only with a peptide of M_r 43,000 having a pI of 7.4; this corresponds to ν_1. Using this mAb, we could then determine whether immunoprecipitation of ν_1 also precipitates a polypeptide containing an ATP binding site and protein kinase activity.

When [α-^{32}P]ATP_{ox} was incubated with AcChoR-enriched membranes in the presence of the reducing agent cyanoborohydride, the labeled ATP_{ox} was covalently linked to ATP binding proteins. One-dimensional $NaDodSO_4$/PAGE (Fig. 2) shows that the major protein(s) labeled by this procedure has a molecular weight of 43,000. A polypeptide at M_r 90,000 is also labeled by [α-^{32}P]ATP_{ox} and is probably Na^+/K^+-ATPase (13). This is the same labeling pattern we obtained with the photoaffinity label 8-azido-ATP (1). Under the conditions of this experiment, the polypeptides of M_r 43,000 are not resolved from one another. We were not able to use two-dimensional electrophoresis of the labeled membranes to identify the M_r 43,000 polypeptides, due to a shift in pI caused by the charged ATP_{ox} moiety. We therefore used immunoprecipitation with the ν_1-specific mAb to determine whether ν_1 binds ATP and has protein kinase activity.

When covalently labeled membranes were solubilized in 0.5% NP-40 and immunoprecipitated by mAb and Pansorbin, only one covalently labeled polypeptide of M_r 43,000 was observed on $NaDodSO_4$/PAGE of the immunoprecipitate (Fig. 3). Control mouse IgG yielded no immunoprecipitated band. Because of the specificity of the mAb, we conclude from these experiments that ν_1 binds ATP.

To demonstrate that the ATP binding protein precipitated by the mAb is a protein kinase, we determined that protein kinase activity was also precipitated from solution by the mAb. The pH 11 extract was neutralized and incubated with either mAb or control mouse IgG, and Pansorbin was added to precipitate antigen–antibody complexes. After centrifugation to separate the immunoprecipitate, the supernatant was evaluated for protein kinase activity. Essentially all of the protein kinase activity in the pH 11 extract was precipitated by the anti-ν_1 antibody (Fig. 4).

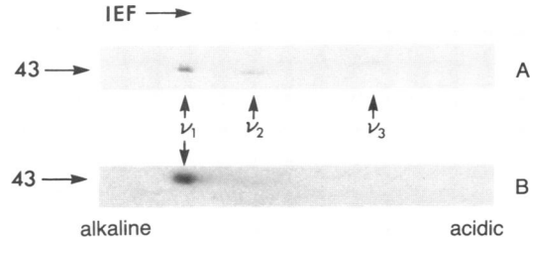

FIG. 1. Specificity of the anti-ν_1 mAb. Neutralized pH 11 extract (15 μg of protein) was subjected to two-dimensional isoelectric focusing (IEF)/$NaDodSO_4$/PAGE. Immunoblotting with a 1:200 dilution of mAb (180 μg/ml) was carried out as described in *Materials and Methods*. (*A*) India ink staining of nitrocellulose transfer. (*B*) Autoradiogram. Only the M_r 43,000, pH 8.0–5.0 region is shown.

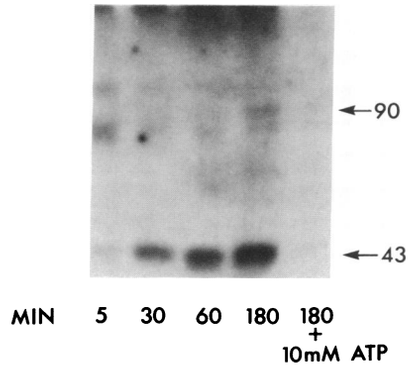

FIG. 2. Time course of [α-^{32}P]ATP_{ox} labeling of AcChoR-enriched membranes. Twenty micrograms of AcChoR-enriched membranes was incubated for the indicated times with 0.5 μM [α-^{32}P]ATP_{ox} in the presence of sodium cyanoborohydride. In one case, the mixture contained 10 mM unlabeled ATP. The reaction mixtures were analyzed by $NaDodSO_4$/8% PAGE (1) followed by autoradiography. Numbers at right indicate $M_r \times 10^{-3}$.

AcChoR-enriched membranes from *Torpedo* electrocyte postsynaptic membranes contain several M_r 43,000 polypeptides. However, only ν_1 is a component of the postsynaptic membrane. We have shown that a mAb to ν_1 immunoprecipitates a polypeptide which binds ATP. Moreover, this mAb also precipitates protein kinase activity from the pH 11 extract of AcChoR-enriched membranes. We therefore conclude that ν_1 is a protein kinase.

Since the M_r 43,000 proteins appear to aggregate in solution, it is possible that the immunoprecipitates could contain a protein kinase(s) of different molecular weight that was trapped in the aggregate. To eliminate this possibility, we carried out the following experiment. Neutralized pH 11 extracts were labeled with ^{125}I by use of lactoperoxidase and then incubated sequentially with mAb and Pansorbin. Two-dimensional gel electrophoresis and subsequent autoradiography of the dried gel showed radioactivity only at M_r 43,000. Therefore, no protein kinase of a higher molecular weight was coprecipitated.

Creatine kinase (ν_2) and actin (ν_3) both have ATP binding

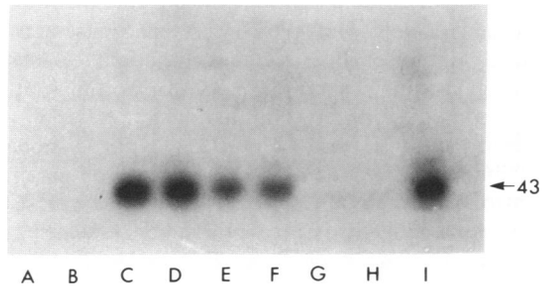

FIG. 3. Immunoprecipitation of labeled M_r 43,000 polypeptide. AcChoR-enriched membranes (20 μg) were labeled with [α-^{32}P]ATP_{ox} and immunoprecipitated with mAb or control IgG and Pansorbin. The immunoprecipitates were analyzed by $NaDodSO_4$/8% PAGE and autoradiography. Lanes: A and B, 10 μl of control mouse IgG (250 μg/ml); C and D, 10 μl of mAb (180 μg/ml); E and F, 10 μl of a 1:10 dilution of mAb; G and H, 10 μl of a 1:100 dilution of mAb; I, labeled membranes in extraction buffer.

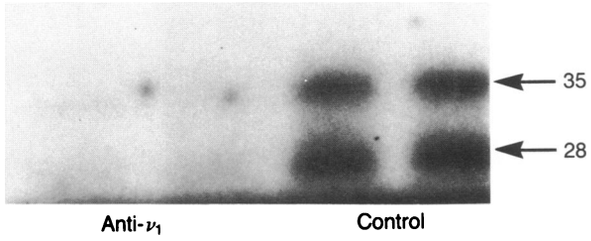

FIG. 4. Protein kinase assay. Neutralized pH 11 extract (0.5 mg of protein per ml) was immunoprecipitated with mAb or control mouse IgG. Supernatants were assayed for protein kinase activity with casein by $NaDodSO_4$/PAGE and autoradiography. The bands at M_r 28,000 and 35,000 are casein polypeptide subunits.

sites and might also be coprecipitated by anti-ν_1 antibody. If either of these proteins had protein kinase activity, it would be difficult to identify ν_1 as the immunoprecipitated protein kinase. The two-dimensional electropherogram of the immunoprecipitate from an ^{125}I-labeled pH 11 extract discussed above showed that 10% of the actin in the pH 11 extract was coprecipitated. This could not account for the loss of 100% of the protein kinase activity from the supernatant. In this experiment, we could not clearly resolve ν_1 from creatine kinase, and so, to eliminate any contribution from creatine kinase to the protein kinase activity, we carried out the following experiments. Both the rabbit muscle and rabbit brain forms of creatine kinase (Sigma) were tested for their ability to phosphorylate casein under the conditions used in our protein kinase assay; no protein phosphorylation by creatine kinase was demonstrable. Additionally, we measured creatine kinase activity (19) before and after immunoprecipitation with either mAb or control antibody under conditions where all the protein kinase activity was precipitated; there was no loss of creatine kinase activity from the supernatant. We therefore conclude that coprecipitation of actin and creatine kinase could not account for the observed loss of protein kinase activity after immunoprecipitation.

Antibodies to ν_1 react almost exclusively with the innervated surface of *Torpedo* electrocytes (9) and crossreact with a component of the rat neuromuscular junction that is highly concentrated at the synapse (9, 20). Both ν_1 (20), which we have shown here to be a protein kinase, and the AcChoR receptor kinase (12) are located on the cytoplasmic side of the postsynaptic membrane. In addition, both ν_1 and the receptor kinase can be extracted from AcChoR-enriched membranes by mild alkali treatment (12, 21, 22). Although it is clear that ν_1 phosphorylates casein, it remains to be determined whether the M_r 43,000 protein kinase is the enzyme that phosphorylates the AcChoR. However, ν_1 protein kinase activity is completely inhibited by detergents required to keep the AcChoR in solution. Therefore, AcChoR phosphorylation by ν_1 must be assayed in reconstituted membranes.

Kennedy *et al.* (23) and Kelly *et al.* (24) have shown that the major "postsynaptic density" protein of mammalian brain also is a protein kinase. This kinase, in contrast to the AcChoR kinase (4, 5), is a calmodulin-dependent enzyme. The finding that major postsynaptic membrane proteins in brain and in electric organ are protein kinases suggests that protein phosphorylation may be important for postsynaptic function. In *Torpedo*, the major substrate *in situ* for protein kinases appears to be the AcChoR. However, alkali extraction of AcChoR-enriched membranes, which removes ν_1, does not affect either the binding of cholinergic ligands or agonist-induced changes in binding affinity and ion permeability (21, 22). It seems more likely that the M_r 43,000 kinase and AcChoR phosphorylation play a role in the clustering or stabilization of the AcChoR at the synapse. Isolation, identification, and purification of this postsynaptic membrane protein kinase now make it possible to test this hypothesis.

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