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Acetylcholine Receptors from Torpedo and Electrophorus Have Similar Subunit Structures†

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ABSTRACT: Previously, acetylcholine receptor purified from the electric organs of electric eels (*Electrophorus electricus*) and electric rays (*Torpedo californica*) (torpedo) had appeared to differ in subunit structure. Receptor from torpedo has the subunit structure $\alpha_2\beta\gamma\delta$, but subunits corresponding only to α, β, and γ had been observed in receptor from eel. Here we report that if membrane fragments of eel electric organ are prepared and detergent extracted in the presence of iodoacetamide, then receptor purified from the extract contains a fourth subunit. Using monoclonal antibodies as well as conventional antisera, we show that the newly recognized subunit of receptor from eel corresponds to the δ subunit of torpedo. A monoclonal antibody to the δ subunit of torpedo cross-reacts with the γ subunit and shows a similar cross-reaction between the δ' and γ' subunits of receptor from eel, indicating the presence of an unexpected structural similarity. Although the function of the β, γ, and δ subunits remains unknown, these results support the concept that receptors from the electric organs of several species and probably also from muscle share a similarly complex subunit structure.

The subunit structure of acetylcholine receptor (AcChR)[1] has been debated (Sobel et al., 1978), but it is now clear that AcChR purified from the electric organ of the marine ray *Torpedo californica* (torpedo) is composed of four subunits (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1976; Chang & Bock, 1977; Lindstrom et al., 1978) in the mole ratio $\alpha_2\beta\gamma\delta$ (Damle & Karlin, 1978; Lindstrom et al., 1979a; Reynolds & Karlin, 1978). The subunits of *T. californica* are four similarly acidic glycopeptides (Lindstrom et al., 1979a; Vandlen et al., 1979) which can be distinguished by apparent molecular weight (38×10^3, 50×10^3, 57×10^3, and 64×10^3 for α, β, γ, and δ, respectively), peptide maps (Froehner & Rafto, 1979; Lindstrom et al., 1979a; Nathanson & Hall, 1979), and antisubunit sera (Claudio & Raftery, 1977; Lindstrom et al., 1978, 1979b). At least part of each subunit is exposed on the extracellular surface of the membrane (Lindstrom et al., 1978, 1979b). *T. californica* AcChR's, perhaps unlike AcChR from other species, exist primarily as dimers bound by disulfide bonds between their δ subunits (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977, 1979; Weitzman & Raftery, 1978). α subunits contain the acetylcholine binding site (Karlin et al., 1976). The functions of β, γ, and δ subunits are unknown. However, since highly purified membrane fractions containing only AcChR retain full ion channel activity (Neubig et al., 1979; Wu & Raftery, 1979) as does AcChR solubilized and purified under conditions which prevent denaturation of the ion channel and then reconstituted into artificial membranes (Anholt et al., 1980; Lindstrom et al., unpublished experiments; Nelson et al., 1980), one or more of these subunits must be involved in the structure of the ion channel whose opening is regulated by acetylcholine binding.

AcChR purified from the freshwater teleost *Electrophorus electricus* (eel) by the same affinity chromatography procedure used to purify AcChR from torpedo appears to contain three subunits (Karlin & Cowburn, 1973) corresponding to α', β', and γ' (Lindstrom et al., 1979b). α' has an apparent molecular weight similar to that of torpedo α and also contains the acetylcholine binding site (Karlin et al., 1976). β' and γ' have apparent molecular weights similar to those of β and γ (Lindstrom et al., 1979b). Antisera to torpedo subunits have been used to show that α', β', and γ' correspond immunochemically to the α, β, and γ chains of torpedo AcChR (Lindstrom et al., 1979b). No component cross-reacting exclusively with antibodies to torpedo δ chains was observed in these experiments.

Here we report that this apparent difference in structure between AcChR from the electric organs of torpedo and eel results from the loss of δ' chains during normal purification procedures. We found that when we included iodoacetamide (IAA) during the initial phases of purification of AcChR from eel, a procedure which helps protect disulfide bonds between the δ chains of torpedo from interchange (Chang & Bock, 1977), we observed a fourth subunit in the purified AcChR similar in molecular weight to δ. This procedure with eel organ did not result in the preservation of AcChR dimers linked by disulfide bonds between δ subunits, as it does with torpedo electric organ. But we were able to use monoclonal antibodies

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[1] Abbreviations used: AcChR, acetylcholine receptor; [^{125}I]-α-BGT, ^{125}I-labeled α-bungarotoxin; torpedo, *Torpedo californica*; eel, *Electrophorus electricus*; $NaDodSO_4$, sodium dodecyl sulfate; IAA, iodoacetamide; MG, myasthenia gravis; EAMG, experimental autoimmune myasthenia gravis.

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and conventional antisera to identify the new subunit as the analogue of torpedo δ. This observation supports other evidence, which will be briefly discussed, suggesting that functionally similar nicotinic AcChR's, including those from muscle, probably all share a similarly complex subunit structure.

Experimental Section

Eel AcChR was purified by affinity chromatography on toxin–agarose (Lindstrom & Patrick, 1974) by the same method used to purify torpedo AcChR (Lindstrom et al., 1978). Frozen electric organ tissue was homogenized for 2 min in a Waring Blendor in 2 volumes of 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, 10 mM NaN_3, and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid plus or minus 10 mM iodoacetamide. After homogenization, 100 mM phenylmethanesulfonyl fluoride in 2-propanol was added to a final concentration of 1 mM. After centrifugation for 1 h at 10000 rpm in a Beckman JA10 rotor, the crude membrane pellet was rehomogenized in 2 volumes of the above buffer mix. Then 10% Triton X-100 was added to a final concentration of 2%. Extraction and purification of AcChR were as previously reported (Lindstrom et al., 1978) without the further use of iodoacetamide. Triton X-100 was exchanged for sodium cholate while the AcChR was adsorbed to toxin–agarose, and AcChR was eluted in solutions containing 0.2% cholate.

AcChR (140 μg) was labeled with ^{125}I after dialysis for 3 days against water to remove cholate and NaN_3. It was lyophilized in a microfuge tube and then resuspended in 30 μL of 0.1 M sodium phosphate buffer, pH 7.5, and 0.1% $NaDodSO_4$. It was then labeled with 4 mCi of ^{125}I by using the solid-phase lactoperoxidase method of David (1971). At 0 °C 10 μL of ^{125}I was added followed by 5 μL of lactoperoxidase conjugated to agarose at 50 mg/mL (a gift from Gary David). H_2O_2 (5.24 μL of $1/10^4$ dilution) was added to initiate the reaction. Then the tube was shaken for 30 min. The reaction was terminated with 10 μL of 1 M NaN_3, and then a crystal of NaI was added to displace free ^{125}I and 50 μL of 2% $NaDodSO_4$ was added to ensure solubilization. Free ^{125}I was removed by chromatography over Sephadex G-25 (1.5 × 10 cm column in 100 mM NaCl, 10 mM sodium phosphate, pH 7.5, and 0.1% $NaDodSO_4$). At 76% incorporation this gave $\sim 1.6 \times 10^{19}$ cpm/mol of AcChR. Stock solutions contained 1 mg/mL BSA, 0.5% Triton X-100, 0.1% $NaDodSO_4$, 100 mM NaCl, 10 mM sodium phosphate, pH 7.5, and 10 mM NaN_3.

Immunoprecipitation of ^{125}I-labeled AcChR used aliquots of 1 mL diluted to contain 1×10^7 cpm. Aliquots of serum (5 μL) or medium from anti-AcChR-producing hybridoma cell lines (≤100 μL) were incubated with the ^{125}I-labeled AcChR overnight. Antisera to subunits of torpedo AcChR from the group E previously described (Lindstrom et al., 1979b) were used. Monoclonal antibodies no. 7, 8, and 11 of those previously described (Tzartos & Lindstrom, 1979) were used, and the bound ^{125}I-labeled antigens were precipitated by using anti-IgG coupled to agarose as previously described (Lindstrom et al., 1979b). The ^{125}I-labeled antigens were solubilized from the washed precipitates with $NaDodSO_4$, electrophoresed, and autoradiographed to identify the bound antigens (Lindstrom et al., 1979b).

Cloned cell lines producing antibodies to AcChR were produced by fusing spleen cells from rats immunized with torpedo AcChR and AcChR subunits with mouse myeloma cell lines using poly(ethylene glycol) according to the methods of Köhler & Milstein (1976). These hybridoma cell lines and some of the properties of the anti-AcChR antibodies they produce are described elsewhere (Tzartos & Lindstrom, 1979).

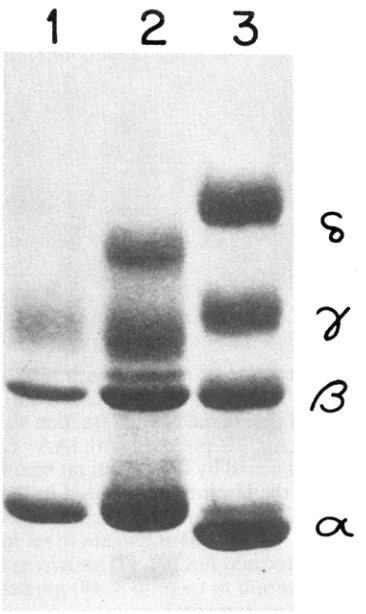

FIGURE 1: Subunit structure of eel and torpedo AcChR. Samples were dissociated in $NaDodSO_4$, electrophoresed, fixed, and stained with Coomassie Brilliant Blue. (1) Eel AcChR as normally purified consists of α′, β′, and γ′ bands. (2) Eel AcChR prepared using IAA also contains a δ′ band. (3) Torpedo AcChR consists of α, β, γ, and δ bands.

Results

AcChR purified from eel electric organ is normally resolved by electrophoresis on acrylamide gels containing $NaDodSO_4$ into three bands. However, when iodoacetamide (IAA) is present in the buffers during the initial phase of purification, electrophoresis of the purified AcChR reveals four bands reminiscent of the four bands observed with AcChR purified from torpedo (Figure 1). The apparent molecular weights of these polypeptides approximate 41×10^3, 50×10^3, 55×10^3, and 62×10^3, as compared to 38×10^3, 50×10^3, 57×10^3, and 64×10^3 for the α, β, γ, and δ subunits of torpedo AcChR. All four bands from eel AcChR stain with the Schiff reagent, suggesting that each contains carbohydrate, as do the torpedo subunits (Lindstrom et al., 1979b) (data not shown).

Previously we had used antisera to each of the torpedo subunits and ^{125}I-labeled polypeptides purified from eel AcChR to determine that the three bands normally observed in eel AcChR correspond immunochemically to the α, β, and γ subunits of torpedo (Lindstrom et al., 1979b). In order to determine whether the fourth band observed after use of IAA corresponded to δ, we used a similar approach. However, instead of purifying each eel AcChR subunit and then labeling with ^{125}I, we labeled whole eel AcChR with ^{125}I to very high specific activity. Incorporation was especially intense in the γ′ chain and least in the δ′ chain (Figure 2). The subunits were dissociated in $NaDodSO_4$ and diluted in Triton X-100. Then the mixture of subunits was allowed to interact with antisera to intact AcChR from eel and torpedo and antisera to torpedo subunits. The antibodies were immunoprecipitated along with the ^{125}I-labeled subunits they bound. Then the solubilized precipitates were electrophoresed and autoradiographed to identify the bound ^{125}I-labeled subunits. Antisera to α and β subunits of torpedo AcChR cross-reacted with α′ and β′ chains of ^{125}I-labeled eel AcChR, as expected (Figure 2, lanes 7 and 8). Antisera to γ reacted primarily with γ′, whereas antisera to δ reacted with both γ′ and δ′ (Figure 2,

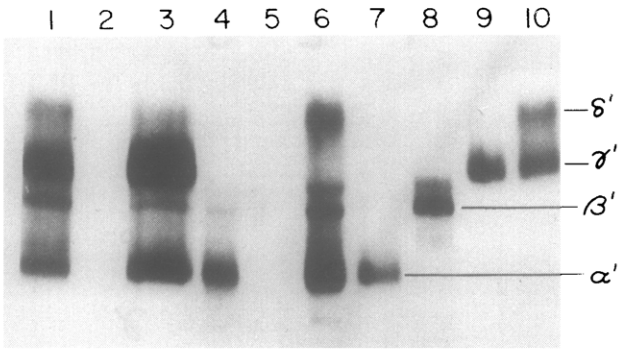

FIGURE 2: Cross-reaction of antisera with subunits of eel AcChR prepared in IAA. Autoradiogram (exposed 24 h) of an acrylamide gel on which 50000 cpm/band of each fraction was electrophoresed. (1) ^{125}I-Labeled eel AcChR prepared with IAA. (2–10) ^{125}I-Labeled eel AcChR subunits bound by (2) normal rat serum (a 50-μL aliquot was used, equal to the largest volume used in the other fractions), (3) antiserum to eel AcChR not prepared with IAA, (4) antiserum to fetal calf muscle AcChR, (5) antiserum to rat muscle AcChR, (6) antiserum to native torpedo AcChR, (7) antiserum to torpedo AcChR α subunit, (8) antiserum to torpedo β, (9) antiserum to torpedo γ, and (10) antiserum to torpedo δ.

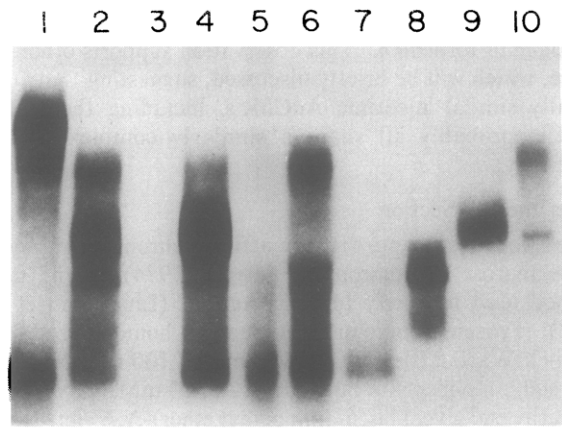

FIGURE 3: Cross-reaction of monoclonal antibodies with subunits of eel AcChR prepared in IAA. This autoradiogram was prepared like that in Figure 2, though with a different preparation of [^{125}I]AcChR. (1) ^{125}I-Labeled α and δ subunits of torpedo AcChR; (2) ^{125}I-labeled eel AcChR; ^{125}I-labeled eel AcChR subunits bound by (3) normal rat serum, (4) antiserum to native eel AcChR, (5) antiserum to fetal calf AcChR, (6) antiserum to torpedo AcChR, (7) monoclonal antibody to torpedo α subunit, (8) monoclonal antibody to torpedo β subunit, (9) antiserum to torpedo γ subunit, and (10) monoclonal antibody to torpedo δ subunit.

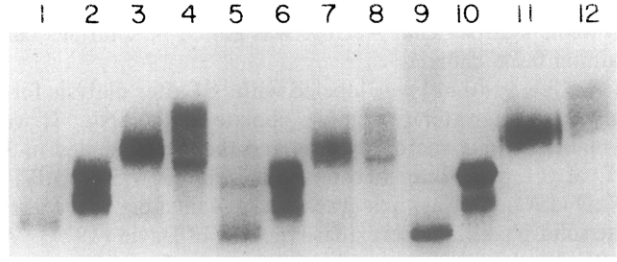

FIGURE 4: Cross-reaction of antitorpedo AcChR subunit antibodies with ^{125}I-labeled eel AcChR prepared with IAA (1–4), ^{125}I-labeled eel AcChR prepared without IAA (5–8), and ^{125}I-labeled purified α', β', and γ' subunits (9–12). ^{125}I-Labeled eel AcChR + IAA was the same batch used in Figure 2 but several weeks older. ^{125}I-Labeled eel AcChR prepared without IAA was iodinated by the same method. α', β', and γ' units were purified from AcChR prepared without IAA and iodinated as previously described (Lindstrom et al., 1979b). The anti-γ-subunit serum and monoclonal anti-α, -β, and -δ antibodies used in Figure 3 were used with each of these three sets of antigens, and bound antigens were identified by electrophoresis and autoradiography as in Figure 3. (1–4) Anti α, β, γ, and δ against ^{125}I-labeled eel AcChR prepared with IAA; (5–8) anti α, β, γ, and δ against ^{125}I-labeled eel AcChR prepared without IAA; (9–12) anti α, β, γ, and δ against an equal molar mixture of ^{125}I-labeled eel α', β', and γ'.

lanes 9 and 10). This suggests that the newly recognized band does indeed correspond to the δ subunit of torpedo and in addition that there is an antigenic (i.e., structural) similarity between γ' and δ'. Other experiments confirm this hypothesis.

Previously we showed that antisera to γ drawn early in the course of immunization react specifically with γ, but after more prolonged immunization they also react with δ. Although this could have been caused by contamination of the γ immunogen by δ, we were able to show that this was not the case. Cross-reaction of γ and δ was explained (Tzartos & Lindstrom, 1979) by showing that monoclonal antibody to δ also had affinity for γ, which implied that γ and δ share a similar antigenic determinant. Antisera to γ drawn early after immunization do not cross-react with γ', whereas after prolonged immunization cross-reaction is observed (Lindstrom et al., 1979b). This result suggests that the antibodies to γ subunits which cross-react with δ subunits are the same antibodies which cross-react between species with γ' subunits. This was confirmed by using as a highly specific probe a monoclonal antibody to δ that has low affinity for γ. Like antisera to δ (Figure 2, lane 10), it reacted with both γ' and δ' subunits (Figure 3, lane 10). Thus γ' and δ' subunits of eel AcChR, like γ and δ subunits of torpedo AcChR, share an unexpected structural similarity. Monoclonal antibodies to α and β subunits confirmed the identities of α' and β' subunits (Figure 3, lanes 7 and 8) observed by using antisera to α and β.

Figure 4 compares the reaction of antisera to torpedo subunits with ^{125}I-labeled eel AcChR purified by using IAA, ^{125}I-labeled eel AcChR purified without using IAA, and a mixture of ^{125}I-labeled purified eel α', β', and γ' subunits. Note that in these experiments antibodies are incubated with large excesses of ^{125}I-labeled antigens; thus, traces of δ' subunit are detectable even in eel AcChR purified without IAA, although the amount of this subunit is not normally sufficient to be detected by Coomassie Brilliant Blue. Note also that the β' subunit shows some breakdown on storage into a fragment with an apparent molecular weight intermediate between those of α' and β'. This closely resembles the spontaneous breakdown of torpedo β into a fragment of similar size (Lindstrom et al., 1978, 1979b).

The use of very high specific activity ^{125}I-labeled whole eel AcChR was not only an easier method for studying interspecies cross-reaction of antisera than purifying AcChR subunits, labeling with ^{125}I, and then studying the binding of antibodies (Lindstrom et al., 1979b) but also more sensitive. We have previously shown (Lindstrom et al., 1979b) that only a small fraction of the antibodies obtained when native eel or torpedo AcChR's is used as immunogens bind to $NaDodSO_4$-denatured ^{125}I-labeled subunits. Although studies with antisera to denatured subunits reveal that AcChR's from both torpedo and eel are composed of four comparable subunits, cross-reaction of antisera to native torpedo AcChR is confined largely to the α' subunit (Lindstrom et al., 1979b). This is in part because an antigenic determinant on α dominates the immunogenicity of native torpedo AcChR in rats (Tzartos & Lindstrom, 1979). However, the very high specific activity AcChR used in these experiments permits detection of cross-reaction with β' and δ' as well (Figure 2, lane 6). No cross-reaction with γ' is observed, which is especially striking because its specific activity is highest, so reaction would be most easily detected. As previously observed (Lindstrom et al., 1979b), antisera to

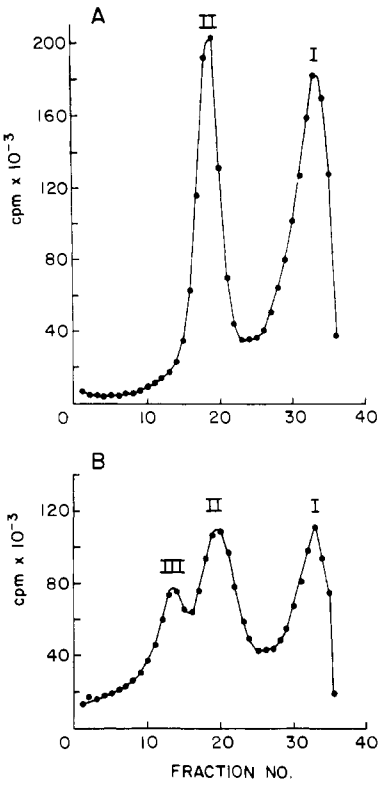

FIGURE 5: Sedimentation of eel AcChR prepared with IAA on sucrose gradients. AcChR at 10^{-7} M was incubated overnight with [^{125}I]-α-BGT, and then aliquots (100 μL) were layered on 4.9-mL 5–20% sucrose gradients and centrifuged for 7 h at 5×10^4 rpm in a Beckman SW50.1 rotor as previously described (Lindstrom & Patrick, 1974). (A) Eel AcChR prepared with IAA. Peak I is excess unbound [^{125}I]-α-BGT. Peak II is AcChR monomer labeled with [^{125}I]-α-BGT. (B) A crude extract of torpedo AcChR initially prepared in IAA was aged for several weeks to permit breakdown of some dimers. Peak I is unbound [^{125}I]-α-BGT. Peak II is AcChR monomer. Peak III is AcChR dimer.

AcChR purified from fetal calf muscle cross-react primarily with α' chains, but slight cross-reaction with β' is also observed (Figure 2, lanes 3 and 4). Although AcChR from fetal calf muscle contains antigenic determinants comparable to α, β, γ, and δ which can be detected by using antisera to torpedo subunits (Lindstrom et al., 1979b), antisera to native calf muscle AcChR, like those to native torpedo AcChR, appear to be directed primarily at determinants on α. The lower titer of the antiserum of calf AcChR (6.1×10^{-6} M) as compared to that of the antiserum to torpedo AcChR (4.3×10^{-5} M) limits the possibility of detecting cross-reaction with subunits other than α by using antisera to calf AcChR.

In order to investigate whether eel AcChR's are dimerized through disulfide bonds between δ' chains like torpedo AcChR (Chang & Bock, 1977), we sedimented eel AcChR purified by using IAA on sucrose gradient (Figure 5). Only monomers were observed, as with eel AcChR purified in the conventional way. The use of 10 mM *N*-ethylmaleimide instead of IAA also did not result in dimers. Thus, either eel AcChR is not dimerized like torpedo AcChR or IAA prevents the loss of δ' but not of δ'–δ' disulfide bonds. Torpedo AcChR dimerization, for example, is destroyed by very mild proteolysis (Lindstrom, 1976).

Discussion

The mechanism by which the use of IAA in the buffer in which eel electric organ is initially homogenized preserves the δ' subunit in AcChR subsequently purified from the electric organ homogenate is not clear. It may act by alkylating a critical thiol on a protease that would otherwise cleave δ' at several sites. The proteolyzed δ' subunit might remain associated with the AcChR macromolecule, so that its sedimentation in sucrose gradients would not be altered. However, if the purified AcChR was dissociated in $NaDodSO_4$ and subjected to electrophoresis, the proteolyzed fragments of δ' might be unrecognizable.

The subunit structure of AcChR from electric organs of torpedoes and eels is similar in many respects. These similarities are (1) close apparent molecular weight of four corresponding subunits, (2) presence of carbohydrate on all subunits, (3) specific affinity labeling of α and α' subunits, (4) immunological cross-reaction of α and α' subunits, (5) immunological cross-reaction of β and β' subunits, (6) spontaneous breakdown pattern of β and β' subunits, (7) immunological cross-reaction of γ and γ' subunits, (8) immunological cross-reaction of δ and δ' subunits, and (9) cross-reaction of anti-δ monoclonal antibody with both γ and γ'. AcChR purified from muscle (Froehner et al., 1977a,b; Nathanson & Hall, 1979) contains chains similar in molecular weight to the four chains composing torpedo and eel AcChR, and muscle AcChR (Lindstrom et al., 1978, 1979b) is also known to contain four sets of antigenic determinants comparable to α, β, γ, and δ. But it is not yet known whether the four chains in muscle AcChR correspond to the four sets of antigenic determinants.

The evidence suggests that AcChR's have retained a very complex subunit structure over broad evolutionary distances, which indicates that these subunits must have important functions. α-Type subunits from electric organ (Karlin et al., 1976) and muscle AcChR (Froehner et al., 1977a,b; Lindstrom et al., 1979b) clearly form at least part of the specific acetylcholine binding site, but other subunits might participate in the structure of this site, the regulation of its affinity through desensitization, or the transduction of acetylcholine binding into the conformation change of the protein which opens the ion channel. Functions of subunits certainly include forming the structure of ion channel, and may include forming the structure of a component which interacts with intracellular filaments (Heuser & Salpeter, 1979) to localize AcChR at the tips of postsynaptic membrane folds, or the structure of a component signaling the rapid extrajunctional or slow junctional turnover rate of the molecule (Fambrough, 1979), or the structure of a component such as a ligand binding site for interacting with intersynaptic components or other ligands.

Acknowledgments

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Effects of Sodium and Lithium Ions on the Potassium Ion Transport Systems of *Escherichia coli*†

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ABSTRACT: The effects of the cations choline, Li^+, and Na^+ on the TrkA and Kdp K^+ transport systems in *Escherichia coli* were studied by observing the accumulation of $^{204}Tl^+$ and K^+. Tl^+ uptake via the TrkA system was stimulated by Na^+ but not Li^+ when compared to choline. A similar effect was observed for K^+ transport via the TrkA system. On the other hand, Tl^+ uptake via the Kdp system was stimulated more by Li^+ than by Na^+ when compared to choline. In addition, Li^+ enhanced the effectiveness of Rb^+ as an inhibitor of Tl^+ uptake via the Kdp system. Na^+, however, was a more effective stimulator of K^+ transport via the Kdp system than Li^+. We suggest that Na^+ may be involved in the mechanisms of K^+ transport via the TrkA and Kdp systems in *E. coli*.

The Na^+/K^+-ATPase of animal cells couples the accumulation of K^+ and the extrusion of Na^+ to hydrolysis of ATP, allowing the cells to maintain intracellular levels of K^+ higher than those found outside (Glynn & Karlish, 1975). In *Escherichia coli* four K^+ uptake systems have been described (Rhoads et al., 1976). Of those, the TrkA and Kdp systems are quantitatively the most important. The other two systems, TrkD and TrkF, are quantitatively minor (Rhoads & Epstein, 1978). The constitutive TrkA system, a low-affinity, high-velocity system, is responsible for the majority of K^+ uptake by wild type strains growing in the presence of millimolar or higher levels of K^+. It requires both ATP and a protonmotive force for activity (Rhoads & Epstein, 1977). The high-affinity repressible Kdp system allows cells to grow at K^+ concentrations as low as 1 μM and is ATP driven (Rhoads & Epstein, 1977). Three structural proteins of the Kdp system have been identified, and two of them have molecular weights similar to those of the two subunits of animal Na^+/K^+-ATPase (Laimins et al., 1978). According to Laimins et al. (1978), however, one difference between the two ATPases is that the Kdp system did not appear to require Na^+.

We have previously shown that *E. coli* accumulates $^{204}Tl^+$ via the TrkA and Kdp systems and proposed that $^{204}Tl^+$ could be used as a probe in the study of such systems (Damper et al., 1979). Some initial observations during that work led us to study the effects of several monovalent cations on Tl^+ and K^+ accumulation via the TrkA and Kdp systems. We show here that Na^+ stimulates the transport of both Tl^+ and K^+ via each of these systems.

Experimental Procedure

Bacterial Strains. Two strains of *E. coli* K-12 were used. TK1001 (*trkA*$^+$, *kdp*$^-$, *trkD*$^-$) (Rhoads et al., 1976) was used

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