Covalent Labeling of the Acetylcholine Receptor from *Torpedo* Electric Tissue with the Channel Blocker [^{3}H]Triphenylmethylphosphonium by Ultraviolet Irradiation†

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ABSTRACT: The lipophilic cation [^{3}H]triphenylmethylphosphonium, frequently used as a voltage sensor in membrane systems, binds reversibly to a site different from the acetylcholine binding site. This is concluded from the different pH dependences of the binding of these two ligands. Furthermore [^{3}H]triphenylmethylphosphonium, previously identified as a channel blocker, can be covalently incorporated into acetylcholine receptor-rich membranes from *Torpedo* electric tissue by UV irradiation of the receptor–ligand complex. In the absence of effector, predominantly the α-polypeptide chains (M_r 40000) of the receptor protein are labeled by the radioactive ligand. The agonist carbamoylcholine strongly stimulates the labeling, but it directs the label predominantly to the δ- and β-polypeptide chains. The antagonist D-tubocurarine and the virtually irreversible competitive antagonist α-bungarotoxin have qualitatively the same effect as the agonist carbamoylcholine. Significant differences were obtained with receptor-rich membranes prepared from *Torpedo marmorata* and *Torpedo californica*: No agonist- or antagonist-stimulated reaction was observed with the latter. The results are interpreted as an indication of a rearrangement of the receptor's quaternary structure caused by cholinergic effector binding preceding discrimination between agonists and antagonists.

The nicotinic acetylcholine receptor consists of at least two functional subunits, the ligand binding moiety and the cation channel, the latter interacting with the former presumably in the same way as regulatory and catalytic subunits in certain enzymes (Heidmann & Changeux, 1978). The effector-binding moiety of the receptor from *Torpedo californica* has been identified by affinity labeling as the α-polypeptide chains of the receptor–protein complex (Reiter et al., 1972; Hucho et al., 1976; Damle et al., 1978). Since the pentameric quaternary structure of the *Torpedo* receptor is $\alpha_2,\beta,\gamma,\delta$ (Reynolds & Karlin, 1978; Hucho et al., 1978; Raftery et al., 1980; Hucho, 1981), the functions of three more polypeptide chains remain to be determined.

Several attempts to identify the cation channel by reversible (Eldefrawi et al., 1977; Sobel et al., 1978) and irreversible (Oswald et al., 1980) binding of "noncompetitive antagonists", presumed to be selective blockers of the ion channel, failed to yield unambiguous results. Recently, we have described a reversible blocker of the receptor ion channel with rather promising properties (Lauffer & Hucho, 1982). Here we show that this blocker, the lipophilic cation triphenylmethylphosphonium (TPMP$^+$),[1] so far known to membrane biochemists as a "voltage sensor", can be activated by irradiation to react covalently with its binding site or membrane components situated close by. The labeling is interpreted as an indication of a quaternary structure change caused by cholinergic effectors, both agonists and antagonists, and not as a reaction with the ion channel itself.

Materials and Methods

Preparation of Membrane Fragments from Torpedo Electric Tissue. Microsacs from *Torpedo marmorata* and *Torpedo californica* were prepared as described (Schiebler & Hucho, 1978), recovered from sucrose gradients by dilution in H_2O (5-fold) and sedimentation (35000g/90 min). Membrane fragments were then resuspended in sodium Ringer solution and left for equilibration overnight in ice. Protein was determined according to Lowry et al. (1951). Vesicles were then diluted to a protein concentration of 1.00 mg/mL with N_2-deaereated sodium Ringer solution.

Reagents. α-Bungarotoxin was from Boehringer, Mannheim, and D-tubocurarine and TPMP$^+$ bromide were obtained from Sigma. [^{3}H]TPMP$^+$ iodide (specific radioactivity 57.4 Ci/mmol) was purchased from New England Nuclear; for use the reagent was evaporated to dryness and redissolved in the 3-fold volume of N_2-deaereated sodium Ringer solution. The final concentration was now 330000 cpm/μL. All other reagents were of the highest purity commercially available.

[^{3}H]Acetylcholine Binding. Binding was measured at 20 °C by an equilibrium centrifugation assay. The membrane suspension (protein concentration 0.1 mg/mL) was incubated for 30 min at room temperature with 10^{-4} M eserine to block acetylcholinesterase. After 15-min incubation with varying Ach concentrations [(0.4–4.5) × 10^{-7} M], 165-μL aliquots were centrifuged for 30 min in a Beckman airfuge. Before and after centrifugation 50-μL aliquots were taken and counted in 5 mL of Supertron (Kontron, Munich) in a liquid scintillation counter to obtain the total and free concentrations of [^{3}H]acetylcholine, respectively. The specific activity of the acetylcholine stock solution (New England Nuclear) was 50 mCi/mmol.

[^{3}H]TPMP$^+$ Binding, Ultrafiltration Assay. The method was basically the same as described before (Lauffer & Hucho, 1982): A membrane suspension (0.1 mg/mL protein, 3500 nmol/g of protein [^{125}I]-α-bungarotoxin binding capacity) in 10 mM acetate or phosphate, pH as indicated in Figure 1, containing 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 0.1 mM phenylmethyanesulfonyl fluoride was used for the ultrafiltration assay. Binding was measured at 20–22 °C. The filters used (EHWP 02500 Celotate filters, pore width 0.45 μm, Millipore, Neu-Isenburg) were soaked for 2 h in the same

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[1] Abbreviations: [^{3}H]TPMP$^+$, [^{3}H]triphenylmethylphosphonium; AchR, nicotinic acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate.

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buffer as above but contained 10 mg/mL bovine serum albumin. Binding reaction was started by 10-fold dilution of the membrane suspension into 10 mM buffer (pH and buffer indicated in the figure; 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mg/mL bovine serum albumin) containing 25 nM [^{3}H]TPMP$^+$ of specific activity 57.4 Ci/mmol. After 5 min 100 μL of the reaction mixture was diluted into 3 mL of ice-cold buffer (composition as above, 1 mg/mL bovine serum albumin) and rapidly filtered by suction. The filter was washed twice with 3 mL of ice-cold buffer (composition as before), and radioactivity was measured with 0.2 mL of H_2O and 5 mL of Supertron (Kontron, Munich) in a liquid scintillation counter.

Covalent Incorporation of [^{3}H]TPMP$^+$ into Receptor-Rich Membrane by UV Irradiation. The procedure was essentially as described (Oswald & Changeux, 1981a,b) with some modifications. Before use, sodium Ringer solution was extensively deaereated with N_2 (30 min). The vesicle suspension (33 μL) in sodium Ringer solution was mixed with 11 μL of [^{3}H]TPMP$^+$; cholinergic effector in sodium Ringer solution and/or further sodium Ringer solution was added to a final volume of 66 μL; effector concentration was 10^{-4} M, except for α-bungarotoxin, which was 10^{-5} M. The incubation mixtures were left for equilibration in ice for 8 min. For experiments with α-bungarotoxin the receptor-rich microsacs were preincubated at room temperature for 30 min. Carbamoylcholine and [^{3}H]TPMP$^+$ were added subsequently, and the mixture was left for binding equilibration as described above. For UV irradiation, the whole sample mixture was placed on a cover slide which was mounted on an ice-cooled steel plate. Irradiation was for 2 min with a prewarmed (30 min) UV lamp (Quarzlampen GmbH, Hanau, Germany, type 5241, 0.35 W) without any filter positioned 8 cm above the sample. Then two aliquots of 30 μL were taken, diluted 1:1 with Laemmli electrophoresis sample buffer (final protein concentration 15.0 μg/60 μL) and left at room temperature for 30 min. To one sample was added lipid crimson (Serva, Heidelberg) to 0.1%. $NaDodSO_4$ electrophoresis with disc slab gels was performed overnight at 3 mA, 4 °C. Acrylamide concentration in the gel was 10% and in the stacking gel 3%. After the bromophenol blue marker and lipid stain were about 2 cm from the lower edge of the gel, electrophoresis was completed. After being stained with Coomassie blue in 30% 2-propanol and 10% acetic acid in H_2O for 1 h, the gels were destained with 30% methanol and 10% acetic acid in H_2O for about 2 h, washed in distilled water, photographed, and prepared for fluorography with sodium salicylate (Sigma) as described (Chamberlain, 1979). Gels were dried and exposed for 7 days to a preflashed Kodak XR5 film in a Kodak X-ray cassette with intensifying screens, type "regular". Disc electrophoresis in 6-mm cylindrical gels at the same acrylamide concentration as above was performed for about 3 h with 3 mA/tube until the lipid and bromophenol blue marker were approaching the lower end. After removal from the glass tubes, the gels were fixed, stained and destained as described above, equilibrated with distilled H_2O, and scanned at 550 nm. Gels were then frozen with dry ice and cut into 2-mm slices. The slices were put into a scintillation vial, solubilized in 5 mL of toluol/Omnifluor (4 g/L), containing 3% Protosol (New England Nuclear), and counted in a Kontron MR 300 scintillation counter (^{3}H channel).

Results

pH Dependence of TPMP$^+$ and Acetylcholine Binding. To obtain maximal TPMP$^+$ incorporation we first investigated the pH dependence of reversible ligand binding to the AchR-rich membrane microsacs. Figure 1 shows that between pH 7 and pH 5 both [^{3}H]TPMP$^+$ binding and acetylcholine binding decrease drastically, indicating participation of functional groups with pK values of about 6 in this binding. Binding is optimal around pH 7–8. Conspicuously, on the alkaline side of the pH optimum the pH dependence is very different for the two ligands. This hints at different binding sites for the channel blocker TPMP$^+$ and the agonist acetylcholine.

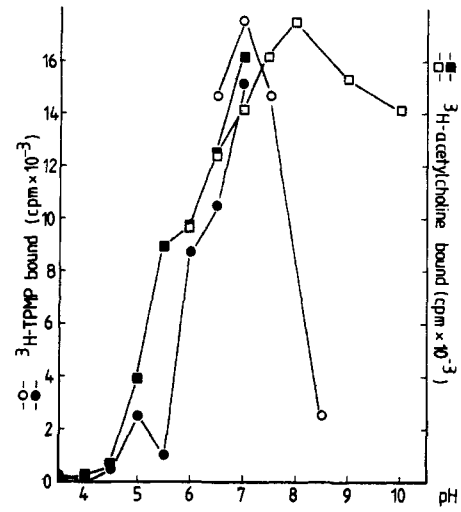

FIGURE 1: pH dependence of [^{3}H]TPMP$^+$ binding (○, ●) and [^{3}H]acetylcholine binding (□, ■): open symbols represent experiments performed in 10 mM acetate and closed symbols in 10 mM phosphate buffer. [^{3}H]TPMP$^+$ binding was determined by an ultrafiltration assay (Lauffer & Hucho, 1982). [^{3}H]Acetylcholine binding was measured by ultracentrifugation at room temperature with an airfuge. Specific radioactivities were [^{3}H]TPMP$^+$ 57.4 Ci/mmol and [^{3}H]-acetylcholine 50.0 mCi/mmol. Receptor protein concentration was 0.1 mg/mL. Specific binding activity of the receptor-rich membranes was about 3500 nmol of [^{125}I]-α-bungarotoxin/g of protein. For acetylcholine binding the AchR-rich membranes were preincubated at room temperature for 30 min with 10^{-4} M eserine to block trace amounts of acetylcholinesterase present in the preparation.

Covalent Incorporation of [^{3}H]TPMP$^+$ by UV Irradiation. Figure 2b shows an autoradiogram of a $NaDodSO_4$–polyacrylamide gel of AchR-rich membranes from *Torpedo marmorata* after 2 min of UV irradiation in the presence of [^{3}H]TPMP$^+$. Track 1 shows one strong band with an R_f value corresponding to the α-polypeptide chain (M_r 40 000). This chain reacts with [^{3}H]TPMP$^+$ in the absence of any other effector. In the presence of the agonist carbamoylcholine (10^{-4} M) the pattern of the labeled bands changes drastically (track 2). Much less label is incorporated into the α-polypeptide chain; instead, heavy labeling of the β and the δ chain is obtained. In addition two new bands corresponding to apparent molecular weights of 120 000 and 130 000 become visible. These molecular weights indicate possibly cross-linking of receptor polypeptide chains by [^{3}H]TPMP$^+$. They correspond perhaps to a δ–δ and a β–δ dimer, respectively. Activation of the receptor by the agonist obviously stimulated labeling of all these polypeptides, which is in accordance with our previous observation (Lauffer & Hucho, 1982) that reversible [^{3}H]TPMP$^+$ binding is strongly stimulated by agonists. The third track shows UV labeling in the presence of carbamoylcholine after preincubation with 10^{-5} M α-bungarotoxin. Labeling of the β and δ chains is diminished probably because of the competitive block of the carbamoylcholine binding site by the toxin. Interestingly [^{3}H]TPMP$^+$ incorporation into the β- and δ-polypeptide chains is also stimulated by the antagonists D-tubocurarine (10^{-4} M) and flaxedil (not shown here).

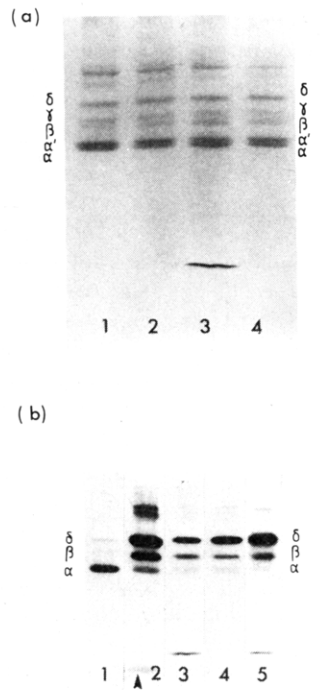

FIGURE 2: Covalent labeling with [^{3}H]TPMP$^+$ by UV irradiation (*Torpedo marmorata*). (a) Coomassie blue stained NaDodSO$_4$–polyacrylamide gel. (b) Autoradiogram (fluorography). (Track 1) AchR/[^{3}H]TPMP$^+$ mixture, irradiated for 2 min in the absence of effector (for experimental conditions, see Materials and Methods). (Track 2) Same as track 1, but in the presence of 10^{-4} M carbamoylcholine. (Track 3) Same as track 2, but preincubated with 10^{-5} M α-bungarotoxin, a 10-fold molar excess of toxin over AchR, before addition of agonist. (Track 4) AchR/[^{3}H]TPMP$^+$ mixture irradiated for 2 min in the presence of the antagonist D-tubocurarine (10^{-4} M). (Track 5) Same as track 3, but in the absence of carbamoylcholine. The lowest band in the autoradiogram tracks 3 and 5 represents α-bungarotoxin which obviously also picked up some radioactivity. The very weak lowest band in track 2 represents probably traces of labeled lipid. The top two bands in track 2 represent apparent molecular weights of 120 000 and 130 000, respectively (see text). Calibration proteins omitted from these figures. α, α', β, γ, and δ represent the predominant polypeptides of the AchR-rich membrane, with molecular weights of 40K, 43K, 48K, 58K, and 68K, respectively.

This raised the suspicion that the observed shift of the label from α to δ and β is not specific for agonists. Figure 2, track 5, confirms this assumption: α-bungarotoxin, a strong antagonist, assumed to be purely competitive, also causes labeling of the δ- and β-polypeptide chains, even in the absence of agonist (compare track 3).

In similar labeling experiments with other channel ligands it had been concluded that labeling takes place with the receptor in its desensitized state (Oswald & Changeux, 1981b). Because of the time scale of the experiment (2-min irradiation) which is slower than desensitization, this is difficult to be excluded. But neither D-tubocurarine nor even less α-bungarotoxin can be considered as effectors shifting the acetylcholine receptor to its desensitized state. A further experiment renders the involvement of this state in the observed photoreaction unlikely. Figure 3 shows the UV labeling with [^{3}H]TPMP$^+$ after various times of preincubation with carbamoylcholine. It is obvious that even after 30-min preincubation there is still an increase in the labeling of the δ-polypeptide chain (all other parameters have been kept constant in this experiment, especially the incubation time with [^{3}H]-TPMP$^+$, irradiation time, etc.).

Figure 4a,b shows some of these results in a more quantitative evaluation with sliced cylindrical gels. It also shows a comparison of the [^{3}H]TPMP$^+$ labeling performed under

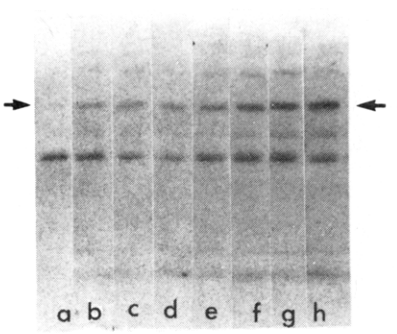

FIGURE 3: Effect of the variation of the time of preincubation with carbamoylcholine on the covalent labeling with [^{3}H]TPMP$^+$. The experiment was performed essentially as described in Figure 2, except that incubation with 1 μM [^{3}H]TPMP$^+$ *after* preincubation with carbamoylcholine (10^{-6} M) and UV irradiation was for 1 min each. Preincubation times with carbamoylcholine: (track a) control (no carbamoylcholine added); (track b) 0 s (carbamoylcholine and [^{3}H]TPMP$^+$ added simultaneously); (track c) 30 s; (track d) 1 min; (track e) 2 min; (track f) 5 min; (track g) 15 min; (track h) 30 min. Watch the absence of radioactivity in δ in track a and the increase of labeling of this band with time (arrow).

otherwise identical conditions with *Torpedo marmorata* (Figure 4A) and *Torpedo californica* (Figure 4B). With the latter [^{3}H]TPMP$^+$ incorporation into the α-polypeptide chain was slightly stronger but no agonist or antagonist stimulation of labeling β and δ chains was observed. The effector-stimulated photolabeling of receptors from *Torpedo marmorata* has been reproduced with more than a dozen membrane preparations. The failure to obtain such labeling was observed with all three preparations of *Torpedo californica* receptor so far investigated. Reversible [^{3}H]TPMP$^+$ binding was found for both *Torpedo californica* and *Torpedo marmorata* (Lauffer, 1983), and receptor-rich membranes from both species were functional in terms of acetylcholine binding and agonist-stimulated ^{22}Na$^+$ efflux.

Discussion

For the nicotinic AchR from *Torpedo* electric tissue, competitive blockers (antagonists) are known which interact with the acetylcholine binding sites and noncompetitive blockers which affect only weakly agonist binding but block instead strongly the cation flux through the receptor-regulated ion channel (Heidmann & Changeux, 1978). The latter group of compounds comprises certain local anesthetics, perhydrohistrionicotoxin (Albuquerque et al., 1973), phencyclidine (Albuquerque et al., 1980), and amantadine (Tsai et al., 1978). We (Lauffer & Hucho, 1982) added to this list of channel blockers the lipophilic cation TPMP$^+$ previously used as a "voltage sensor" which accumulates into membranes in a voltage-dependent manner (Schuldiner & Kaback, 1975). It is assumed that channel blockers exert their effect by binding directly to the cation channel (e.g., Albuquerque et al., 1973). Therefore they are hoped to be used as tools to identify the ion channel within the receptor–protein complex. Especially useful for this purpose would be covalently reacting channel blockers. Several attempts to label the ion channel irreversibly with a radioactive ligand have been made (Blanchard & Raftery, 1979; Oswald et al., 1980; Oswald & Changeux, 1981a, b). All these compounds reacted predominantly with the α- or δ-polypeptide chain of the AchR, especially in the presence of agonists. The conclusion from these observations could be that the agonist is opening the ion channel, rendering it accessible to the channel blocker. The δ-polypeptide chain therefore would be a good candidate for being at least one of the building blocks of the ion channel, if not the ion channel

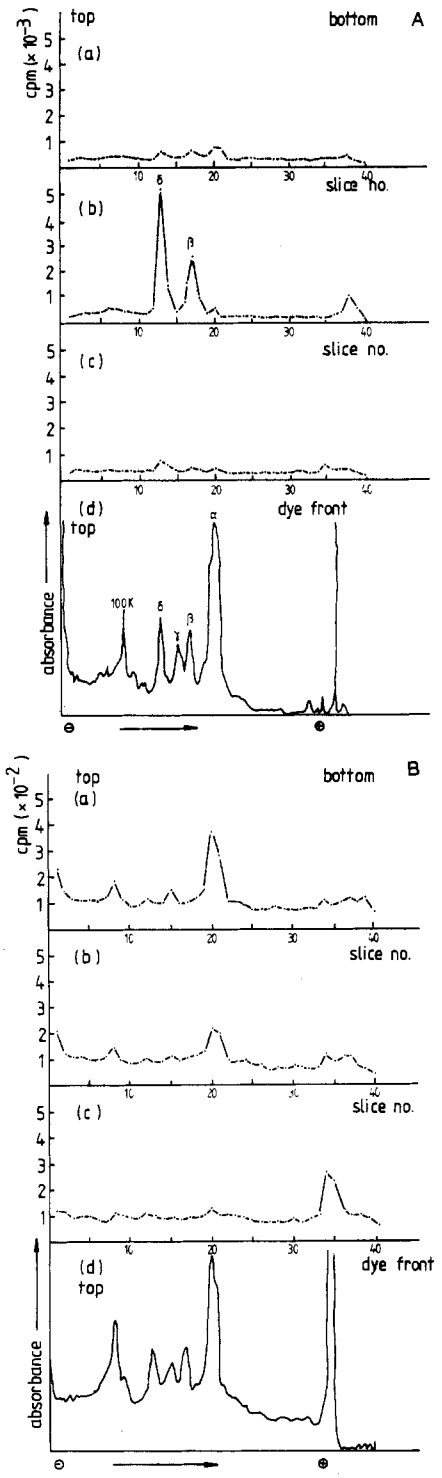

FIGURE 4: Comparison of [^{3}H]TPMP$^+$ labeling of AchR from *Torpedo marmorata* (A) and *Torpedo californica* (B). 10% NaDodSO$_4$–polyacrylamide gels were scanned at 550 nm after Coomassie staining (d). (a) UV irradiation of an AchR/[^{3}H]TPMP$^+$ mixture in the absence of effector. (b) same as (a), but UV irradiation in the presence of the agonist carbamoylcholine (10^{-4} M). (c) same as (b), but with preincubation of the AchR with 10^{-5} M α-bungarotoxin which displaces the agonist and reduces [^{3}H]TPMP$^+$ labeling of the β- and δ-polypeptide chains (*Torpedo marmorata*). Note the absence of radioactivity in these bands in *Torpedo californica*. Gel slices were 2 mm.

itself. But because of the long duration of these labeling experiments, it is most likely that in all cases the receptor had undergone already a transition to its desensitized state, which is characterized by its high affinity for agonists and its impermeability for cations. Therefore it was concluded (Oswald & Changeux, 1981b) that covalent labeling of acetylcholine receptors by noncompetitive blockers takes place not with the open receptor-regulated channel but with the desensitized receptor complex.

We believe that this conclusion is at least premature for the following reasons: (i) The experiments with [^{3}H]TPMP$^+$ presented here show that also antagonists, by definition ligands which bind to the AchR but without opening the cation channel and without desensitizing it, stimulate radioactive labeling of the δ-polypeptide chain. (ii) The time scale of the labeling (minutes) is rather different from the time scale of the channel opening (milliseconds). It may be closer to the time scale of desensitization (seconds to minutes), but this correlation collides with the observation that nondesensitizing antagonists also stimulate reversible (Lauffer & Hucho, 1982) and irreversible (this publication) binding of [^{3}H]TPMP$^+$. Furthermore, the exeriment in Figure 3 shows that carbamoylcholine preincubation of up to 30 min still enhanced photolabeling of the δ-polypeptide chain. Of course the time scale of desensitization is difficult to evaluate precisely in this membrane system, and no data are available which are obtained under strictly identical conditions. But the transition from the resting to the desensitized state should be complete long before 0.5 h. (iii) The different results obtained with AchR-rich membranes from *Torpedo marmorata* and *Torpedo californica* are an argument against the conclusion that the covalent reaction of channel ligands is monitoring an essential process of the ligand-induced channel opening: AchR-rich membranes from both *Torpedo* species are biochemically very similar (Deutsch & Raftery, 1979) and behave basically identical even in their ligand-induced *reversible* binding of [^{3}H]TPMP$^+$. The *irreversible* labeling therefore must be an expression of a property of the receptor–protein not being functionally important for the action mechanism of the receptor complex.

Several positive interpretations can be added to this negative conclusion resulting from our experiments: (i) In the receptor–effector complex the binding site for the channel blocker TPMP$^+$ is located in close proximity to the δ- and the β-polypeptide chains. This conclusion of course implies that the covalent reaction of [^{3}HTPMP$^+$ takes place with the same site on the receptor–protein complex which was previously characterized by reversible [^{3}H]TPMP$^+$ binding (Lauffer & Hucho, 1982). But we are convinced that this is the case, because the covalent labeling is prevented by the noncompetitive antagonist tetracain (not shown here), indicating the specificity of the reaction for the binding site for noncompetitive blockers. (ii) Agonist and to a lesser degree antagonist binding render reactive sites of the protein accessible to [^{3}H]TPMP$^+$ and/or cause a rearrangement of the quaternary structure, locating the δ and β chains in a position favorable for reactions with the UV activated [^{3}H]TPMP$^+$. This favorable position is obviously not obtained with the *Torpedo californica* AchR. (iii) In the presence of agonist even some lipid incorporates radioactivity. This reaction between the photolabel and membrane lipids (or small peptides migrating with the dye front in NaDodSO$_4$–polyacrylamide gel electrophoresis) is (not shown here) not noticeable with freshly prepared receptor-rich membranes but increases strongly with aging (3–4 days storage at 0 °C). With aging also more and more labeling of the δ-polypeptide chain is obtained, even in the absence of any cholinergic effector. (iv) The UV-induced covalent labeling of AchR confirms the conclusions drawn from our reversible [^{3}H]TPMP$^+$ binding experiments (Lauffer & Hucho, 1982): [^{3}H]TPMP$^+$ binds to a cation binding site distinct from the

agonist binding site. This site is exposed by both agonists (carbamoylcholine) and antagonists (D-tubocurarine, α-bungarotoxin) hinting at a structural change in AchR independent of discrimination between agonists and antagonists. The photolabeling with [^{3}H]TPMP$^+$ cannot be correlated with any of the receptor states discussed so far (Changeux, 1981). The widely accepted four-state model of the nicotinic acetylcholine receptor therefore may be an oversimplification.

Added in Proof

The effect of bungarotoxin described in Figure 2, track 5, was most pronounced with a sample from Boehringer. Since this toxin contains a protease and since trypsin causes similar effects, the observed labeling pattern does not necessarily stem from the toxin proper.

Acknowledgments

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Registry No. TPMP$^+$, 15912-74-0; acetylcholine, 51-84-3; carbamoylcholine, 462-58-8; D-tubocurarine, 57-94-3; α-bungarotoxin, 11032-79-4.

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