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SPECIES DIFFERENCES DETERMINE AZIDO PHENCYCLIDINE LABELING PATTERN IN DESENSITIZED NICOTINIC ACETYLCHOLINE RECEPTORS

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Acetylcholine receptor enriched membranes from Torpedo ocellata, Torpedo marmorata and Torpedo californica were studied using [^{3}H] azido-phencyclidine (AZ-PCP). [^{3}H]-PCP binding to receptors from all three species revealed marked similarities. Photoaffinity labeling by [^{3}H]-AZ-PCP resulted in the tagging of mainly α, β and δ subunits in all species. When carbamylcholine was added, it enhanced the labeling of β subunits in T. ocellata, δ in T. marmorata and α in T. californica, suggesting species differences in the photolabeling pattern. Multiple homologous binding sites for PCP between the receptor subunits would allow small variations in receptor structure to be manifested in labeling by AZ-PCP, with no differences in binding and functional properties of the receptors

The acetylcholine receptor (AcChoR) from Torpedo electric organ is a pentamer composed of four different polypeptides with an apparent molecular weight of ∿ 40,000 (α), ∿ 50,000 (β), ∿ 60,000 (γ) and ∿ 65,000 (δ) dalton (1, 2). Each pentamer contains two α subunits, which carry the binding sites for acetylcholine and snake toxins (1, 2). Studies with radiolabeled aminated local anaesthetics (3, 4), perhydrohistrionicotoxin (5) and phencyclidine (PCP) (4, 6-9) indicated that these noncompetitive blockers of the AcChoR bound to high affinity sites distinct from the AcCho binding sites. The agonist carbamylcholine strongly enhanced the binding of these drugs, suggesting that their sites are associated with the cholinergic binding site.

Photolabile derivatives were employed to localize the polypeptide which binds noncompetitive blockers of AcChoR. Blanchard and Raftery (10) have demonstrated labeling of the 40,000 dalton polypeptide in AcChoR enriched membranes from T. californica by procaine azide amide. Oswald and Changeux (4) have demonstrated selective labeling of the δ subunit of the AcChoR from T. marmorata electric organ by azidotrimethisoquine, or PCP (11). The enhanced labeling in-

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duced by carbamylcholine occurred only on the δ subunit. We have recently used azido-phencyclidine to localize PCP binding sites on the AcChoR from T. ocellata electric organ (12). Our results indicated that carbamylcholine induced a specific increase in the labeling of the 50,000 dalton polypeptide. These variations in the labeling of AcChoR subunits could not be attributed to the different ligands used, or to the presence of proteolytic enzymes that could cleave the receptor subunits (4. 12). The enhanced labeling by PCP and AZ-PCP of different subunits in the AcChoR from different species (δ subunit in T. marmorata, β in T. ocellata) suggested species differences as the source of the discrepancies. We have examined this possibility using azido-PCP and receptor enriched membranes from T. ocellata, T. marmorata and T. californica. We report here marked differences in the labeling of the AcChoR subunits from these three species by AZ-PCP in the presence of carbamylcholine.

Materials and Methods

Materials: [^{3}H]-PCP (22.5 Ci/mmole) was prepared by catalytic tritium exchange and tested for purity (> 98%) as described elsewhere (13, 14). [^{3}H]-AZ-PCP (27.1 Ci/mmole) was prepared by diazotation of tritiated aminophencyclidine (9) followed by reaction with sodium azide (15). Purity was > 99%. Tetracaine, carbamylcholine and α-Bgt were obtained from Sigma.

Tissue preparation: Membranes rich in acetylcholine receptor were prepared as described by Saitoh et al (16) from 150 g of freshly dissected electric organ of live T. ocellata or T. marmorata or 150 g of nitrogen frozen tissue of T. californica. The electric organs were homogenized in 50 mM Tris-HCl buffer, pH 7.4, in the presence of 0.1 mM methylmethylsulfonyl fluoride, 3 mM EDTA, 1 mM EGTA, 5 units/ml of aprotinine and 5 μg/ml of pepstatin A. Membranes were purified by fractionation (16) followed by alkaline treatment (17). Membranes were either used immediately or stored in the homogenization buffer at -70°C until use. Specific α-Bgt binding sites were determined by the DEAE filter disk assay (18), using ^{125}I-labeled α-bungarotoxin (^{125}I-α-Bgt). The purified AcChoR rich membrane fragments had a specific activity of 0.8-2.0 nmol/mg protein A typical coomassie blue staining pattern of the membranes is shown in Fig. 1. The four receptor subunits are clearly observed in the gels of all three species. Polypeptides with apparent molecular weight of ∿ 40,000, ∿ 50,000, ∿ 60,000 and ∿ 65,000 dalton, which correspond to the AcChoR from T. californica and T. marmorata, have been well described (1, 2). In a previous work (19) on AcChoR from T. ocellata, a 65,000 dalton polypeptide was not detected. In our preparations of AcChoR enriched membranes from T. ocellata, we have consistently observed this polypeptide in the gels. Other bands of 80,000 and ∿ 93,000 dalton are also present in these preparations (Fig. 1), and similar polypeptides can be observed in preparations from other Torpedo species.

Binding assays: Binding of [^{3}H]-PCP and [^{3}H]-AZ-PCP was assayed by the centrifugation method as described previously (6, 9). The latter ligand was assayed in the dark. All assays were carried out in triplicate together with triplicate samples containing 5×10^{-4} M unlabeled PCP. Specific binding was calculated as the total minus the nonspecific (the latter is the binding in the

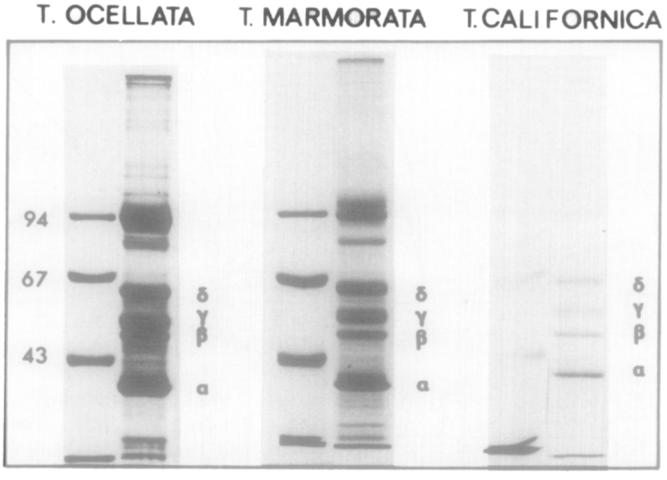

Figure 1

Coomassie blue staining pattern of polypeptides from receptor enriched membranes from T. ocellata, T. marmorata and T. californica electrophoresed in SDS polyacrylamide gel. Membranes were prepared and electrophoresed as described in Methods.

presence of 5×10^{-4} M unlabeled PCP). The dissociation constants (K_d) and the maximal binding capacity (B_{max}) were derived from Scatchard plots by a linear least square regression analysis. Protein was determined by the Lowry (20) method using bovine serum albumin as standard.

Photoaffinity labeling: AcChoR-enriched membranes were incubated with 1.0 μM [^{3}H]-AZ-PCP in the dark for 30 min under conditions similar to those described for the binding assays (6, 9, 12). The incubation was carried out in the presence and absence of various agents. Nonspecific binding was determined in the presence of 1 mM PCP or tetracaine. Following incubation samples were withdrawn for determination of total binding. The reaction mixture was then photolyzed with a long wave ultraviolet spotlight lamp (Thomas Scientific Apparatus, model B-100A), at a distance of 5 cm (1500 μW/cm^2) with continuous stirring for 4 min. After photolysis, membranes were precipitated and washed 4 times with Tris-HCl buffer (50 mM, pH 7.4).

Gel electrophoresis: SDS polyacrylamide gel electrophoresis procedure was carried out as described by Laemmli (21). Membrane samples were dissolved 1:1 (v/v) in 2 x sample buffer (52.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and applied to polyacrylamide slab gels (8.0%). Proteins were electrophoresed at 15-20 mA/slab for 3 h, then stained with 0.2% Coomassie Blue in 50% methanol and 7% acetic acid, and destained in 7.5% acetic acid. Gels were sliced into 1 mm sections with a gel slicer. Each slice was digested in 5 ml of 6% protosol (NEN) in Hydro Luma in a closed scintillation vial. Radioactivity was determined after 24 h by liquid scintillation spectrometry.

Results and Discussion

Data on high affinity binding of [^{3}H]-PCP to AcChoR-enriched membranes from Torpedo ocellata, Torpedo marmorata and Torpedo californica are shown in Fig. 2. The apparent dissociation constants determined with membranes from

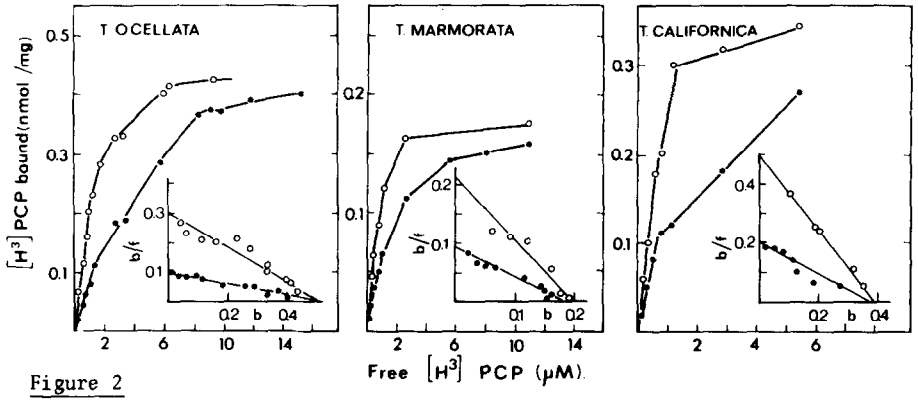

Figure 2

Specific binding of [^{3}H]-PCP to receptor enriched membranes from T. ocellata, T. marmorata and T. californica. Binding was determined as described in Methods in the absence (•) and presence (o) of 10^{-5} M carbamylcholine. Inserts - Scatchard plots: Bound ligand in nmole/mg protein (b) and bound over free ligand (b/f) in (nmole/mg x M) x 10^6. In the membrane preparations used the number of ^{125}I-α-Bgt binding sites (nmole/mg protein) were: 1.5, 0.8 and 1.19 for T. ocellata, T. marmorata and T. californica, respectively.

T. ocellata are 6.9±3.1 μM and 1.7±0.8 μM in the absence and presence of carbamylcholine, respectively. With T. marmorata membranes, the corresponding constants are 3.1±1.3 μM and 0.9±0.4 μM, as compared with 1.4±0.6 μM and 0.5± 0.2 μM in T. californica. Thus, in all cases carbamylcholine induced an increase in the affinity of [^{3}H]-PCP but not in the number of high affinity binding sites. Ratio of PCP/α-Bgt sites is 0.30, 0.25 and 0.28 in T. ocellata, T. marmorata and T. californica respectively (Fig. 2). The binding data are in good agreement with previous results (6-9).

In another report (12) we have demonstrated that the reversible binding characteristics of [^{3}H]-PCP and [^{3}H]-AZ-PCP are very similar. Thus, the azido derivative could be used as a photoaffinity probe for PCP binding sites. SDS polyacrylamide gel electrophoresis was used to identify the polypeptides containing [^{3}H]-AZ-PCP in the AcChoR enriched membranes. Membranes prepared from the three Torpedo species were subjected to electrophoresis after photoaffinity labeling with 1.0 μM [^{3}H]-AZ-PCP. Labeling of various polypeptides by [^{3}H]-AZ-PCP was clearly observed after separation by gel electrophoresis (Fig. 3). In the absence of carbamylcholine, [^{3}H]-AZ-PCP labeled mostly the α, β and δ subunits from all species studied. When membranes were labeled in the presence

1.0 mM PCP, most of the labeling disappeared. This indicates that in their resting state (no carbamylcholine), AcChoRs from T. ocellata, T. marmorata and T. californica are tagged specifically by [^{3}H]-AZ-PCP. Comparison of the gel patterns of receptors from the three species, reveals some variations in the distribution of counts between α, β and δ subunits. However, it is clear that three receptor subunits are labeled in all cases. We therefore conclude that they all possess recognition sites for PCP. The simplest explanation would be that there are three binding sites for PCP on the AcChoR. However, the binding data do not seem to support it. If there were indeed three independent binding sites, the ratio of high affinity PCP binding sites to α-Bgt sites would have been at least 3/2. Since the observed value is ∿ 1/3, clearly no more than one PCP molecule can be attached per receptor molecule. Together with the observation that three subunits are labeled, these findings indicate that at least two polypeptides form the binding site for PCP, which is located between them. The high degree of amino acid sequence homology between the receptor subunits allows formation of a pseudosymetric oligomer (2). Therefore, it is most likely that there are similar recognition sites between pairs of receptor subunits that would bind PCP.

The presence of carbamylcholine induced an increase in the labeling of AcChoR subunits by [^{3}H]-AZ-PCP in each of the species tested (Fig. 3). However, in each case the labeling of a different subunit was increased. In T. ocellata the labeling of the β subunit was enhanced; in T. marmorata, it was the δ subunit, and in T. californica it was the α subunit. Thus species variations are clearly apparent in the presence of carbamylcholine, where the receptor is in its desensitized form (1, 2). Since membranes from electric organs of the three species studied were prepared by the same procedure, the phenomenon described must be related to endogenous factors. In view of the marked similarities between AcChoR's from various species (1, 2), it seems unlikely that mechanisms underlying binding of PCP or local anaesthetics to the AcChoR would vary between species. Indeed, binding of [^{3}H]-PCP and labeling of AcChoR's in the resting state by [^{3}H]-AZ-PCP, indicate close resemblance among the species. As discussed

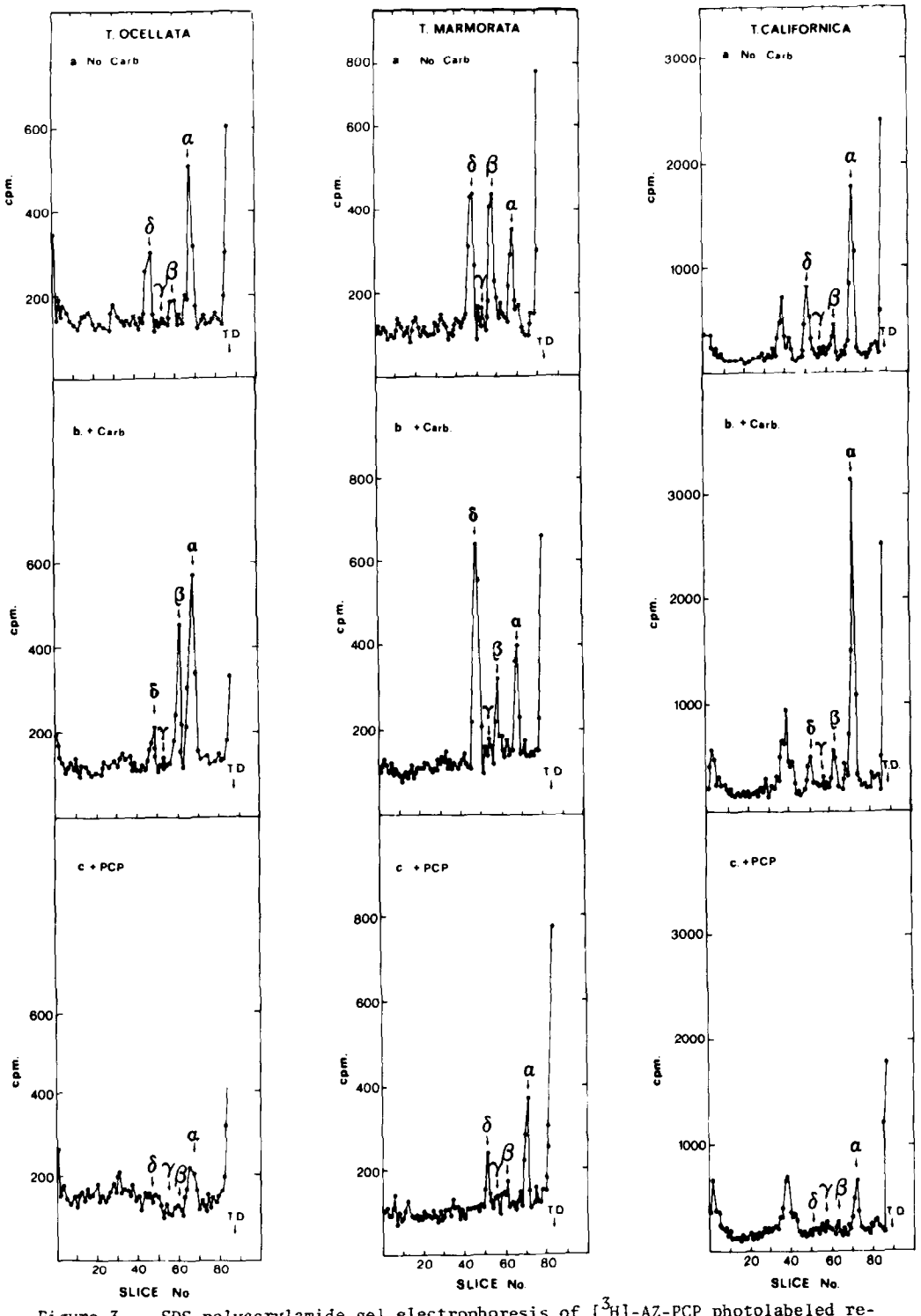

Figure 3 SDS polyacrylamide gel electrophoresis of [^{3}H]-AZ-PCP photolabeled receptor-enriched membranes. Membranes were treated, sliced and counted as described in Methods. Incubations were under the following conditions: a. No carbamylcholine, b. + 100 μM carbamylcholine, c. + 10^{-3} M PCP. Left panel - membranes from T. ocellata; middle panel - membranes from T. marmorata and right panel - membranes from T. californica.

earlier (12), the binding sites for PCP or local anaesthetics (11) are probably located between the receptor subunits. Thus several similar sites could exist in a receptor molecule. Accordingly, occupation of any of these sites by PCP could lead to similar binding and functional activities, but different labeling by AZ-PCP. We suggest that the differences between species described here could stem from multiple homologous binding sites for PCP or local anaesthetics on the AcChoR.

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