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Functional renaturation of receptor polypeptides eluted from SDS polyacrylamide gels

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Abstract. In order to gain further support for the concept that a homo-oligomeric protein-complex may be sufficient to form a functional ligand-activated ion channel and to explore additional possibilities for the reconstitution of channel activity, a single polypeptide band of the purified neuronal AChR from insects has been electroeluted from SDS-polyacrylamide gels, the SDS removed and the polypeptides incorporated into liposomes. Liposomes were fused into planar lipid bilayers which were subsequently analysed for channel activity. Fluctuations of cation-channels were detected after addition of agonists (carbamylcholine); channel activity was blocked by antagonists (d-tubocurarine). The channels formed by electroeluted polypeptides gave conductance values, as well as kinetic data, quite similar to channels formed by the native receptor protein. Sedimentation experiments using sucrose density gradient centrifugation revealed that a considerable portion of the electroeluted polypeptides assembled during the reconstitution process to form oligomeric complexes with a sedimentation coefficient of about 10 S; thus resembling the native receptor complex.

Key words: Electroelution – Membrane transport – Acetylcholine-receptor – Ion channel

Introduction

The nicotinic acetylcholine receptor (AChR) from muscle cells and electrocytes is still the best characterized ligand-activated ion channel; it is constituted of four different polypeptides forming a pentameric complex ($\alpha_2 \beta \tau \sigma$) (Numa et al. 1983; Noda et al. 1983; Montal et al. 1986). Although some specific functions can be associated with one of the subunits, it is still a matter of debate if the basic function of the receptor, i.e. forming ligand-activated ion channels, can be performed by a simpler molecular structure. In contrast to the complex hetero-oligomeric structure of the peripheral AChR, the ligand-activated receptors in the nervous tissue seem to be constituted of only two different polypeptides (Lindstrom et al. 1988). The results of recent expression-experiments using subunit specific mRNAs for neuronal receptor polypeptides suggest that even homo-oligomeric complexes can form functional receptors (Boulder et al. 1987).

Biochemical and reconstitution studies of an α-toxin binding membrane protein from the nervous tissue of locust suggested that this insect ACh-receptor may represent a protein complex of 4–5 identical subunits: $M_r = 65\,000$ (Breer et al. 1985; Hanke and Breer 1986). As there is still some controversey on the subunit structure of this receptor type and also to demonstrate unequivocally that a homo-oligomeric structure can form a functional AChR-channel, attempts have been made to isolate the polypeptides ($M_r = 65\,000$) from SDS-polyacrylamide gels, to remove the SDS quantitatively and then to reconstitute these polypeptides (eventually renatured to functional proteins) into planar lipid bilayers.

Materials and methods

Preparation

The neuronal AChR from insect (*Locusta migratoria*) head and thoracic ganglia was purified by affinity chromatography using α-BTGX coupled to Sepharose 4B as described previously (Breer et al. 1984, 1985). After elution from the affinity column, the protein was lyophylized and stored at $-70°$. For experiments with the native AChR, the detergent was removed by dialysis and the protein incorporated into asolectin liposomes as described previously (Hanke and Breer 1987).

Preparative SDS-page

The concentrated purified material was added to 4 volumes of buffer containing 0.5% SDS, 1% β-mercapto-

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ethanol, 50 m*M* *Tris*-HCl, 12.5% glycine and 0.0013% bromophenolblue. SDS-polyacrylamide gel electrophoresis was performed on a Biometra minigel system with a 5% acrylamide stacking-gel and a 7.5% acrylamide separation-gel at 25 mA for about 90 min. Polypeptide bands were stained with 0.3 *M* $CuSO_4$. The protein band of about 65 kD (determined using a calibration kit, Pharmacia) was cut out and destained with 0.25 *M* EDTA and 0.25 *M* *Tris*-base. Control gels of the native and the renatured receptor were stained with silver.

Electroelution

The destained gel slices were added to 0.5 ml buffer containing 25 m*M* *Tris*, 192 m*M* glycine and 0.1% SDS (electrode buffer) and placed in a dialysis-bag (MWCO 12–14 000). The electroelution was performed for 2 h at 70 mA (as the current is kept constant, the voltage varies) in 10 times diluted electrode buffer with 0.1% SDS. The electroelution was performed in a chamber built by our workshop. The size is 10 cm × 15 cm, the electrodes (steel wire, 0.3 mm diameter) are 14 cm apart and 9.5 cm long. The chamber is filled with buffer so that the dialysis-bag is just covered.

Removal of SDS and incorporation into liposomes

The electroeluted material was incubated for 30 min with TSK-Fractogel DEAE and washed three times with buffer containing 10 m*M* Hepes/KOH, pH 7.4; 1 m*M* $CaCl_2$, 1 m*M* DTT, 10 m*M* CHAPS and 0.2% asolectin (Sigma, partially purified according to Cook et al. 1986) Each time the material was centrifuged and the supernatant was decanted. Finally, protein was removed from the gel by washing with the same buffer containing additional 0.8 *M* KCl and a subsequent centrifugation.

To the supernatant, now containing the protein, the same volume of reconstitution buffer (10 m*M* Hepes/KOH pH 7.4, 1 m*M* DTT, 10 m*M* CHAPS, 10 mg/ml asolectin) was added. The material was then dialysed for 48 h at 4 °C against a buffer containing 100 m*M* KCl, 5 m*M* Hepes/KOH, pH 7.6, 1 m*M* $CaCl_2$, to remove the detergent and to incorporate the polypeptides into liposomal membranes. After dialysis the liposome preparation was kept for three days at 4 °C to enhance reaggregation of the polypeptides in the liposomal membranes.

Density gradient centrifugation

Linear sucrose density gradients (5 to 20%) were prepared as described previously (Breer et al. 1985). A detergent extract (1% deoxycholate) from the proteoliposomes, was layered on top of a preformed sucrose gradient, which was centrifuged for 12 h at 40 000 rpm (Beckman SW-41). After centrifugation, the gradient was fractionated (25 samples) and binding activity for α-BGTX determined in each fraction by incubating aliquots with 5 nM [^{125}I]-α-BGTX for 2 h at 25°, followed by filtration on DE-81 discs (Whatman).

Bilayer reconstitution experiments

Bilayers for experiments with the renatured protein were made from partially purified asolectin (Cook et al. 1986) using the dip-stick technique (Hanke 1985). Liposomes were added to the bath solution and proteins were incorporated into the bilayer by spreading the liposomes at the air-water interface. A bath solution containing 120 m*M* NaCl, 10 m*M* *Tris*/HCl, pH 7.4 and drugs as specified in the results section was used.

Alternatively, folded bilayers according to Montal and Mueller (1972) were used in experiments with the native receptor protein and liposomes were fused into these bilayers as described (Hanke 1985; Hanke and Breer 1987).

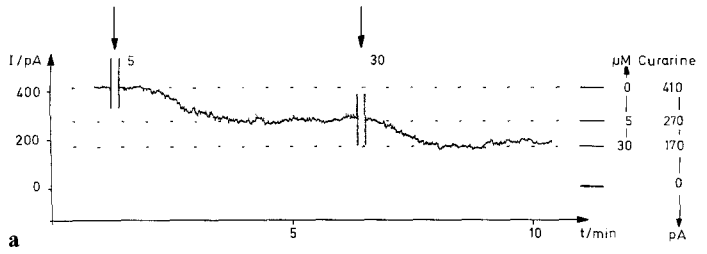
Details of the electrical- and mechanical set-up used in this study have been described elsewhere (Hanke 1985; Hanke and Breer 1986, 1987). A List EPC-7 amplifier was used for the current recordings, data were stored on an FM-tape recorder and subsequently analyzed on an IBM AT-03 computer as described (Hanke and Breer 1987, 1989).

Results

In some first experiments the functional integrity of affinity purified AChR isolated from insect nervous tissue was verified. Figure 1 a shows the response of a multi-channel system in a planar lipid bilayer after activation by 100 μ*M* Carb, followed by a blockade with two different concentrations of d-tubocurarine. Figure 1 b shows a long trace of single channel fluctuations of a neuronal AChR at high Carb concentration (500 μ*M*). For the conditions described in Fig. 1 b the single channel conductance was $\Theta = 75$ pS and the state lifetimes $t_{closed} = 3$ ms and $t_{open} = 21$ ms; the probability of the channel to be in the open state was $P_0 = 0.87$.

The purified and reconstituted receptor protein was analysed by SDS-polyacrylamide gel electrophoresis as described in the methods section. In Fig. 2 a silver-stain of a polyacrylamide-gel is shown, only one polypeptide band ($M_r = 65\,000$) is visualized. In order to evaluate if the ligand-activated channel activity was exclusively due to the polypeptides detected on the SDS-polyacrylamide gel by silver staining, attempts were made to isolate the 65 Kd polypeptides from the gel, to renature the protein and to analyse for its ability to form a ligand activated channel after reconstitution in planar lipid bilayers.

For these purposes, the purified protein was solubilized in SDS and electrophoretically separated on 12.5% slab acrylamide gels. Following electrophoresis, the location of the 65 Kd polypeptides was determined by staining either the detached edges of the gel with silver or alternatively the whole gel reversibly with copper. The localized region of the 65 Kd polypeptide band on the gel



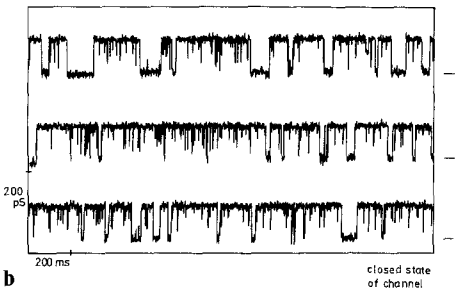


Fig. 1 a, b. Functional properties of the isolated and affinity purified native AChR reconstituted in folded planar lipid bilayers made from asolectine. **a** The conductance of a bilayer containing some ten channel copies was recorded after activation by 100 μ*M* Carb. The channel activity was blocked by d-tubocurarine in a concentration-dependent manner. **b** Single channel fluctuation of a neuronal AChR-channel activated by 500 μ*M* Carb. Single channel parameters were determined as $P_0 = 0.87$, $\Theta = 75$ pS, $t_{open} = 21$ ms and $t_{closed} = 3$ ms, only the insides burst parameters are given

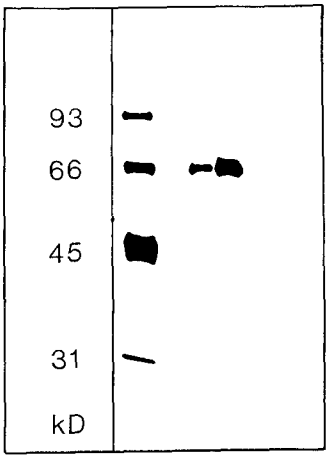


Fig. 2. Polypeptide pattern of the neuronal AChR purified by α-toxin affinity chromatography. Two different concentrations of receptor preparations as well as molecular weight markers (Pharmacia) were electrophoresed on 12.5% SDS-polyacrylamide gel and subsequently visualized by silver staining

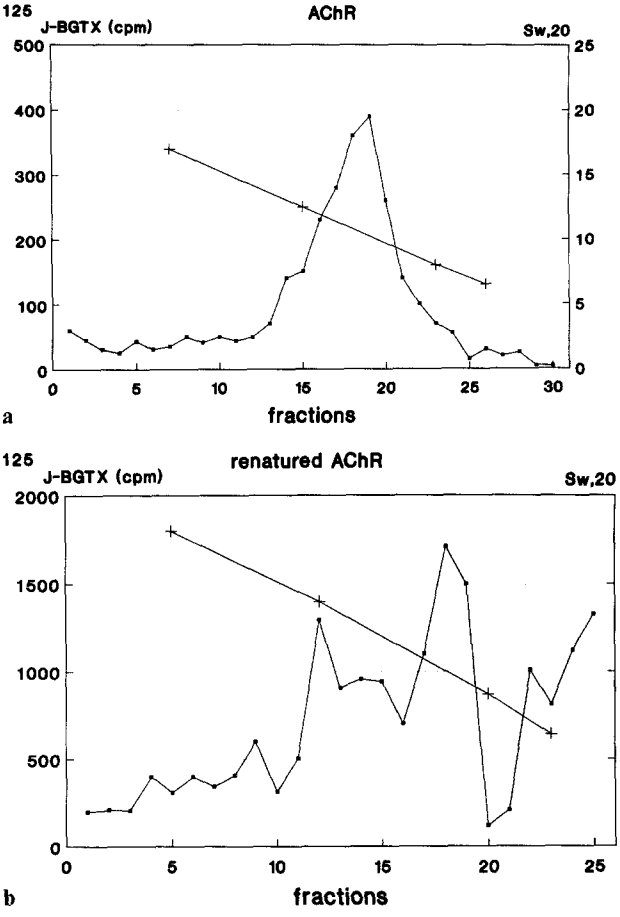


Fig. 3 a, b. Density gradient centrifugation of electroeluted and renatured AChR polypeptides (**b**). The gradient was fractionated into 25 samples. Each fraction was assayed for α-bungarotoxin binding activity. Catalase and phosphatase were used as calibration markers. In **a** the data of the native neuronal AChR from Breer et al. (1985) are shown for comparison. (SW 20 = sedimentation coefficient in Svedberg)

was cut out and the polypeptides were subsequently isolated by electroelution.

Preliminary experiments have shown that not even traces of SDS can be tolerated during reconstitution of the receptor into planar lipid bilayers, as SDS destabilizes the membrane and induces artifical current fluctuations, making the analysis of the ligand induced current very difficult. Thus, every effort including dialysis and incubation with ion exchanger was made to completely eliminate SDS from the eluted protein sample and to exchange the detergent for CHAPS and asolectin. The isolated polypeptides were subsequently incorporated into liposomes using the dialysis technique. After dialysis and storage for several days (at 4 °C) the preparation displayed specific high affinity binding activity for [^{125}I]-α-BGTX, thus indicating that the receptor proteins were at least partially renatured. The restoration of α-toxin binding activity allowed us to explore if the isolated polypeptides had even formed oligomers during the renaturing and reconstitution process. For this purpose the sedimentation of the renatured receptor protein in sucrose density gradients was analyzed. The sedimentation profile seen in Fig. 3 shows that a large portion of the α-toxin binding protein is found in a peak corresponding to a sedimentation coefficient of about 10 S. A very similar sedimentation profile has recently been described for the native receptor protein (Breer et al. 1985). This observation demonstrates that a considerable portion of the electroeluted receptor polypeptide has assembled to form complexes very similar to the native AChR. In order to verify that the complex was in fact only composed of identical

65 Kd polypeptides, the proteoliposomes were extracted with SDS-sample buffer and the extract was analyzed on SDS-polyacrylamide slab gels again. The result obtained was identical to the gel shown in Fig. 2.

After having shown that toxin binding activity as well as the native oligomeric structure can be restored, the function of the renatured protein, i.e. the ligand-activated channel activity, was investigated in planar lipid bilayers (Hanke and Breer 1989). For analysing the channel properties of the renatured receptor polypeptides the dip-stick technique was applied; all other experimental conditions were as given in Fig. 1. Figure 4 shows single channel fluctuations recorded from a bilayer containing only renatured receptor proteins; the channels were activated with two different Carb concentrations. It is obvious that the renatured and reconstituted polypeptides in fact form very distinct ion channels. The fluctuation pattern is clearly agonist concentration dependent (the channel conductance is not affected by the Carb concentration), a pronounced bursting behaviour (Hanke and Breer 1987) can be seen at high Carb. In Fig. 5a the amplitude histogram of the channel conductance at low Carb concentration is presented, giving a conductance of about 75 pS and an open state probability of about 0.09. This conductance step was observed almost exclusively. However, other conductance steps (sublevels) were found with a very low probability, as has been demonstrated with the native receptor-protein (Hanke and Breer 1986, 1987).

The lifetime histograms of the same trace are shown in Fig. 5b; the closed lifetime histogram is a single exponential distributed with a mean closed lifetime (t_{closed}) of

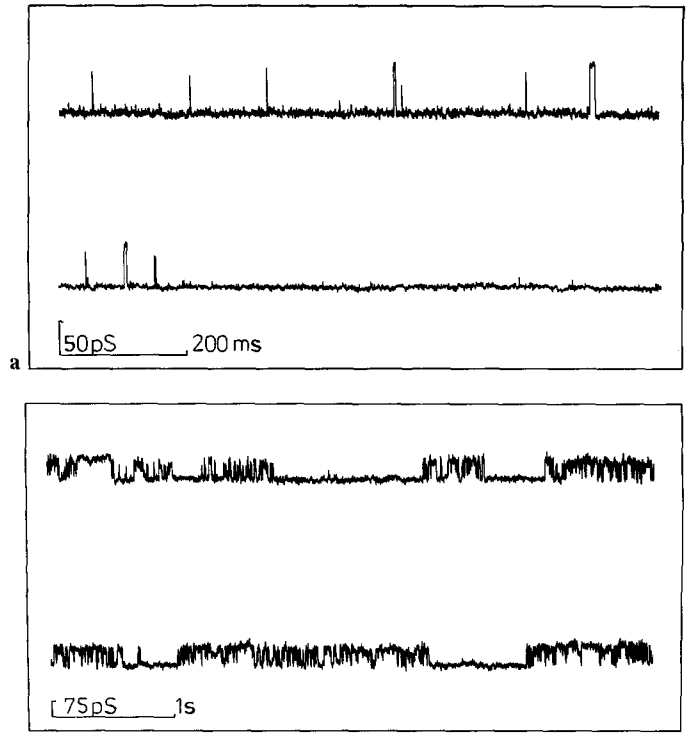


Fig. 4a, b. Single channel fluctuations of renatured AChR incorporated into planar lipid bilayers (dip-stick technique). **a** Fluctuation traces of a single channel at 10 μM Carb. **b** Fluctuation traces of a single channel at 500 μM Carb

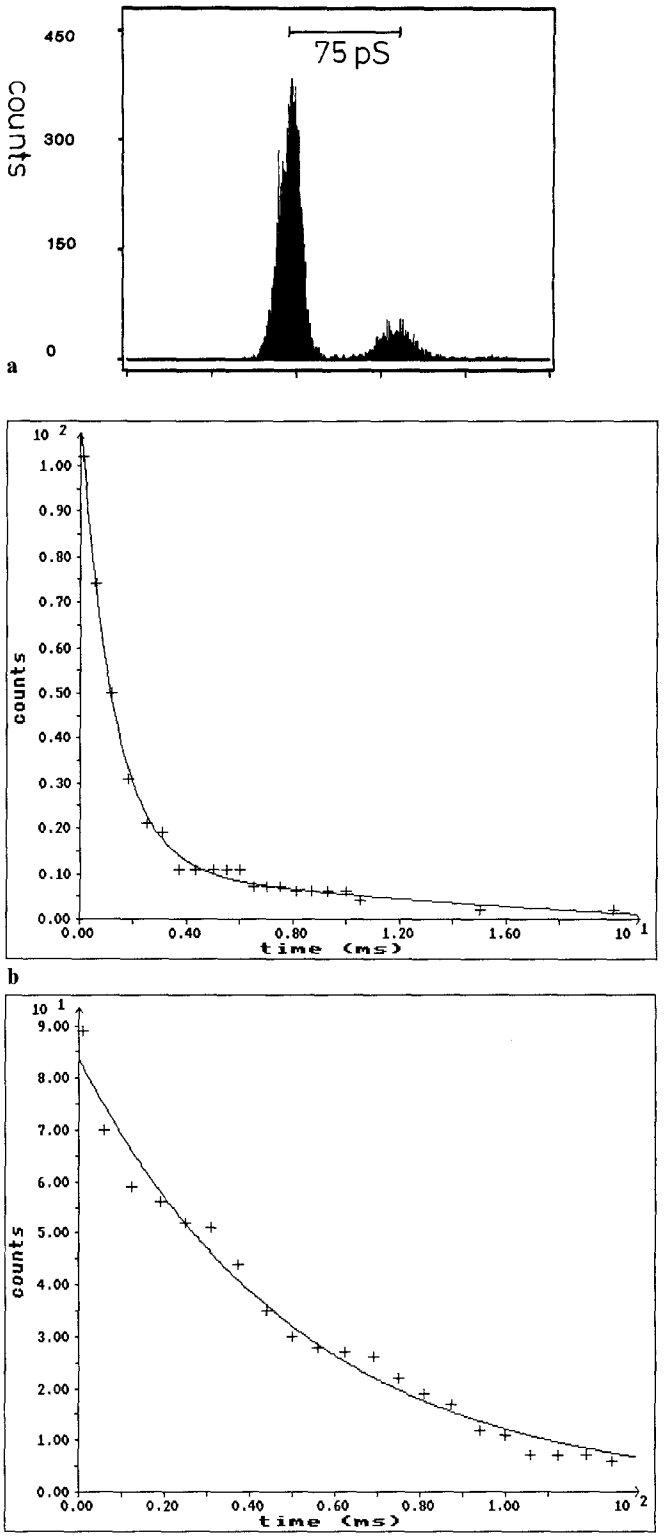


Fig. 5a, b. Basic data evaluation of the single channel fluctuation from Fig. 4a. **a** Amplitude histogram. This clearly exhibits a well-efined closed state and a well-defined open state. The channel conductance determined from the histogram is 75 pS. The probability of the channel being in the open state (P_0) was determined to be $P_0 = 0.09$. **b** The lifetime distributions of both, the open (upper part) and the closed state (lower part) of the channel from above are given. Both distributions are fitted by sums of exponentials as given in the figures. The closed lifetimes are single exponential distributed, resulting in a mean closed lifetime of 70 ms. The open lifetimes can be better fitted by a double exponential decay with $t_{01} = 2$ ms and $t_{02} = 15$ ms. The slow component is about 10% in amplitude

70 ms, the open lifetime histogram can be better fitted by a double exponential decay with (τ_{open1}) of 2 ms and (t_{open2}) of 15 ms, the mean is about 7 ms. The channel parameters evaluated for the renatured receptor complex are apparently very similar, or even identical, to those reported for the native protein (Hanke and Breer 1987, 1989).

Apart from the findings reported above, the spontaneous activity of the receptor (Hanke and Breer 1989), i.e. current fluctuations without agonists being present, was higher. Additionally the bilayers were more noisy with the renatured preparation than with the preparation of the purified native receptor. This may be due to non-reassembled polypeptides present in the preparation (see Fig. 3). In accord with the above statements we were able to find Carb-induced channel activity from renatured AChR-preperations in 8 out of 10 preperations.

To further characterize the renatured receptor, the probability of the channel being in the open state was calculated for different Carb concentrations and plotted in a Hill-plot (at high agonist concentrations only the fluctuations inside bursts were evaluated). In Fig. 6 the results of this analysis are given, together with data obtained for the native AChR. Obviously both sets of data can be fitted with one straight line giving a slope of 0.8.

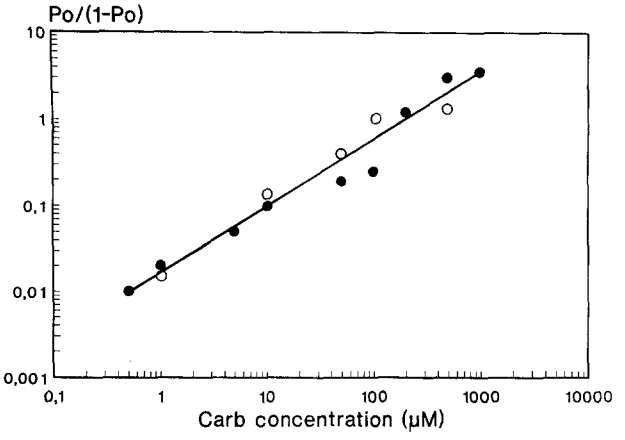


Fig. 6. Hill-plot analysis of the probability to be in the open state for single channel fluctuations (all data at 50 mV). The data for the native AChR (●) and renatured AChR (○) can be fitted by one straight line, giving a slope of 0.8

In addition to the experiments shown, we found that the activity of the renatured AChR in planar lipid bilayers could be blocked by d-tubocurarine at μM concentrations.

From these results we conclude that, in planar lipid bilayers, both the native receptor and the renatured AChR are activated by one agonist molecule and display identical behaviour. For comparison in Table 1 some basic properties of different acetylcholine receptors and different preparations are summarized. The lifetimes of the native and renatured AChR differ somewhat. However, one should remember that channel life-times in bilayer experiments may vary by at least a factor of two from one experiment to another. Furthermore, the value given in the table is a mean of a double exponential fit.

Discussion

The results of the present studies indicate that receptor polypeptides can survive SDS-treatment and can be renatured to form functional ligand-activated ion-channels. Until now, approaches using electroelution of identified polypeptides followed by reconstitution and assaying for functional activity have only be applied successfully to some smaller polypeptides. Noteably, the major polypeptide of gap-junctions, the 27 Kd junctional protein was electrophoretically purified, electroeluted and reconstituted into planar lipid bilayer. It was found that these renatured proteins formed channels with characteristics quite similar to those produced by intact gap junctions (Young et al. 1987); however, these experiments still suffered from incomplete SDS-removal. The authors conclude from their experiments that oligomerization of the monomeric 27 Kd protein may be required for channel formation; but this could not be demonstrated in their study. The recovery of toxin binding activity in the renatured ACh-receptor polypeptide allowed us to monitor their assembly into oligomeric complexes. It is interesting to note that the renatured polypeptides formed oligomers exhibiting the same sedimentation coefficient as the na-

Table 1. Comparison of the properties of different AChR's. All data are given for physiological conditions and low agonist concentration. Data for the peripheral AChR from muscle membranes obtained in patch-clamp experiments are very similar to those for the Torpedo AChR. Patch-clamp experiments with nerve cells of insects gave results similar to those of the native Locusta AChR (single channel conductance 40–60 pS, cooperativity taken from Hill-plot ≥ 1 (Tareiluls, Hanke, Breer, unpublished results)). a-Btx = α-bungarotoxin, Sub = suberyldicholine, Ach = acetylcholine, Carb = carbamylcholine, curare = d-tubocurarine

	Properties of AChR's		
	Torpedo AChR	Native Locusta AChR	Renat. Locusta AChR
Subunit structure	Hetero-oligomer	Homo-oligomer?	Homo-oligomer
Cooperativity	2	1	1
Conductance	50 pS	75 pS	75 pS
Open state lifetime	3 ms	5 ms	7 ms
Potency of agonists	Sub-Ach-Carb	Sub-Ach-Carb	-Ach-Carb
Pharmacology	Block by curare a-Btx binds	Block by curare a-Btx binds	Block by curare a-Btx binds
Spontaneous activity	Very low	Low	High

tive receptor protein (Breer et al. 1985), thus the presumed pentameric structure of the native receptor may be energetically favoured.

Furthermore, the data demonstrate unequivocally that a homo-oligomeric polypeptide complex is sufficient to form a ligand-activated ion channel. This view has gained strong support from current findings made in molecular studies. Marshall et al. (1988) have recently demonstrated that mRNA from a single locust clone microinjected into *Xenopus* oocytes induced the expression of functional acetylcholine receptors. These observations are partially in contrast to results obtained in expression experiments with the hetero-oligomeric *Torpedo* receptor, where all 4 subunit polypeptides were apparently necessary to form fully functional receptors (Numa et al. 1983; Noda et al. 1983). On the other hand there are some recent reports indicating that for several neuronal receptors the expression of α-subunit mRNA induces functional receptors (Pritchett et al. 1988; Blair et al. 1988). These results suggest that the formation of functional receptor channels by homo-oligomeric complexes may be a special feature of neuronal receptors. As the primary structures of several peripheral and neuronal receptors are available, it will be of some interest to explore if the different behaviour of the various receptor types can be attributed to some intrinsic structural differences.

As the possibility cannot be ruled out that differences in the biogenesis and assembly processes of neuronal and peripheral receptors may account for the different behaviour of the receptor types, it will be of considerable interest to apply the new elution and renaturing techniques to the hetero-oligomeric receptor from the electric tissue of *Torpedo*, trying to reconstitute the 4 different receptor subunits separately.

Furthermore, this approach may be extented by using distinct portions of the polypeptides obtained by controlled proteolysis and identified with site-specific antibodies. This approach appears particularly promising as it has been shown very recently, that synthetic peptides which represent part of a functional membrane protein can be reconstituted in planar bilayers. In an elegant study it has recently been demonstrated that a synthetic peptide, which mimics the sequence of the putative transmembrane M2 segment of the *Torpedo californica* acetylcholine receptor δ-subunit, forms discrete ionic channels in phosphatidylcholine bilayers. These channels exhibit features, such as conductance, selectivity and life times, that are characteristic of the authentic AChR channel (Oiki et al. 1988).

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