ACETYLCHOLINE RECEPTOR: -SH GROUP REACTIVITY AS INDICATOR OF CONFORMATIONAL CHANGES AND FUNCTIONAL STATES

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1. Introduction

One of several alternative models of chemical transmission proposes that the conformational state of the acetylcholine receptor (AchR) controls the ion-permeability of the postsynaptic membrane [1–4]. This hypothesis is supported by reports of different states of affinity of the receptor in vitro for cholinergic agonists [5–7]. At least in solution the different states have been shown to be interconvertible [8,9]. The time scale of the interconversion is of the order of minutes and in this respect similar to the phenomenon of pharmacological desensitization. It was postulated that the desensitized state corresponds to a 'high affinity state' of the acetylcholine receptor [9].

In addition to binding studies [8,9] the postulated conformational transition was investigated by fluorescence spectroscopy [10–13]. In this report we show that the reactivity of -SH groups towards Ellman's reagent can be used as an intrinsic indicator of conformational changes and functional states of the receptor: incubation with the agonist carbamyl choline slowly reduces the reactivity. Detergent solubilization of the membrane bound receptor exposes further -SH groups. These are rapidly oxidized in Triton X-100 but remain stable in sodium dodecyl sulfate. Since receptor purification is usually performed in Triton X-100 this finding may explain reported differences between membrane bound and isolated receptor.

In addition to the reported -SH groups a disulfide bridge cross-linking two δ-subunits of the receptor was discovered.

2. Materials and methods

2.1. *Chemicals and reagents*

All compounds were of the highest commercially available purity. Triton X-100, sodium dodecyl sulfate, dithionitrobenzoic acid (Ellman's reagent) and all reagents for polyacrylamide electrophoresis were purchased from Serva (Heidelberg, FRG). Sucrose, ethylenediaminetetraacetate and all buffer substances were obtained from Merck (Darmstadt, FRG).

2.2. *Electric fish*

All experiments were performed with *Torpedo californica* obtained live from Biomarine Supply Inc., Venice, California. The animals were killed immediately before the preparation. Excess electric tissue was frozen with liquid nitrogen, stored at −60°C and used within four weeks.

2.3. *Receptor rich membranes*

Membranes rich in acetylcholine receptor were prepared as described in [14], but with a continuous gradient from 25–50% sucrose and with 0.1 mM phenylmethylsulfonylfluoride present to inhibit proteolysis. The preparations revealed on SDS–polyacrylamide gel electrophoresis predominantly the four bands [15,16] assigned to receptor protein. These membranes were reacted in a Durrum stopped-flow spectrophotometer with 5,5′-dithiobis(2-nitrobenzoic acid) [17].

All other experimental details are included in the legends to the figures.

3. Results and discussion

Figure 1 shows that the reactivity of -SH groups in the membrane decreased significantly when the membranes were preincubated with, e.g., carbamyl choline, an agonist of the cholinergic synapse. The inhibitory effect is not a simple protection of the -SH groups by carbamyl choline because it occurs much slower than the binding of the ligand. (fig.3a, insert). Maximum reduction of the -SH group reactivity occurs after incubation of the receptor with carbamyl choline for more than 20 min. If carbamyl choline only competed with Ellman's reagent for -SH groups the inhibitory effect should be maximal within milliseconds. The time course of the effect can be interpreted as an indication of a slow

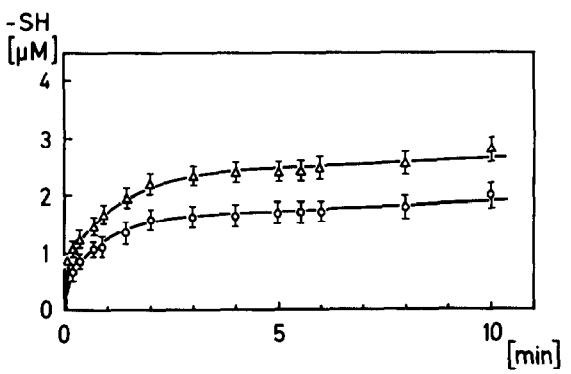


Fig.1. Effect of 50 μM carbamyl choline on the reactivity of -SH groups of AChR-rich membranes. Reaction of -SH groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent, Serva, Heidelberg, FRG) was followed at 412 nm in a Durrum-Gibson stopped-flow spectrophotometer at 25°C. AChR-Rich membranes from the purest fractions of the sucrose gradient [14] were suspended in an equal volume of 0.1 M phosphate buffer, pH 8.0 to give an initial concentration of 0.14 mg protein/ml. (○–○) Carbamyl choline treated membranes: 10 μl of a 0.01 M carbamyl choline solution in H_2O were added to 2 ml of membrane suspension at 25°C. After 40 min preincubation the reaction was carried out against a 1 mM DTNB solution in 0.1 M phosphate buffer, pH 7.0. After 10 min, only a background absorbance increase could be observed. (△–△) Controls: -SH group reactivity was determined before and after the carbamyl choline incubation period, with an aliquot of a non-treated buffered membrane suspension. Every point in the curves represents at least 4 determinations and only 1 out of 8 experiments needed to be discarded due to abnormal drifts of deficient membrane preparation.

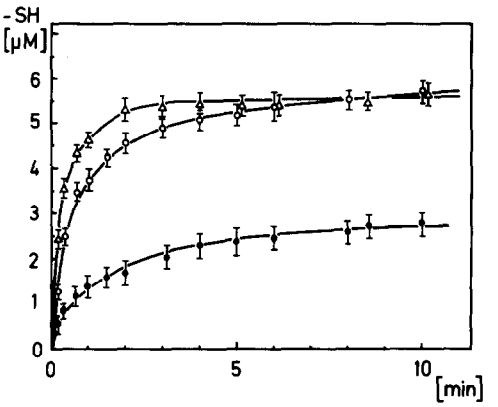


Fig.2. Effect of an ionic and a non-ionic detergent on the reactivity of -SH groups of AChR-Rich membranes. For experimental procedure see legend to fig.1 with the following modifications: (△–△) 1 mM DTNB in 0.1 M phosphate buffer, pH 7.0 with 2% w/v Triton X-100 (Serva, Heidelberg, FRG). (○–○) Same DTNB solution with 1% w/v sodium cholate (Merck, FRG). (●–●) Control as in fig.1.

conformational change caused by the ligand which moves -SH groups of the receptor to a slightly different environment thereby changing its reactivity.

Solubilization of the membrane by detergents (Triton X-100 or sodium cholate) exposes more -SH groups as compared to the intact membrane (fig.2). Triton X-100 and sodium cholate appear to affect the -SH group reactivity differently. This observation may be related to the different affinities of the receptor for, e.g., acetylcholine found in these detergents [8]. In detergent solution -SH group reactivity is also influenced by preincubation with cholinergic agonists (fig.3). Carbamyl choline shows the strongest effect, decamethonium and the antagonist D-tubocurarine is less effective and hexamethonium shows at least in the initial phase of the reaction very little effect.

The α-neurotoxin of *Naja naja siamensis* appears also to influence the -SH group reactivity, but since binding of this ligand is slow we cannot tell whether it exerts its effect via a conformational change or by direct competition with DTNB for -SH groups.

It should be mentioned that the -SH groups of the acetylcholine receptor are not essential for the binding of ligands [13]. But they appear to be situated at a site which interacts with the binding site

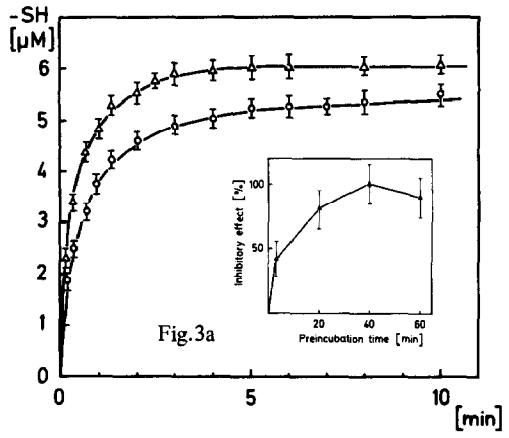


Fig.3a. Effect of 50 μM carbamyl choline on the reactivity of -SH groups of AChR-rich membranes solubilized with Triton X-100. For experimental procedure refer to legend to fig.1. 1 mM DTNB solution/2% w/v Triton X-100 was shot against a membrane suspension with (○) and without (△) carbamyl choline preincubation. Controls as in fig.1. The insert shows the time-dependence of the decrease of reactivity of -SH groups of AChR-rich membranes after different incubation times with 50 μM carbamyl choline in an analogous experiment. Measurements were usually made after 3, 20, 40 and 60 min preincubation. The percentage decrease of the reacted -SH groups 3 min after starting the reaction with DTNB is plotted against preincubation time. Maximum effect was typically after 20 min.

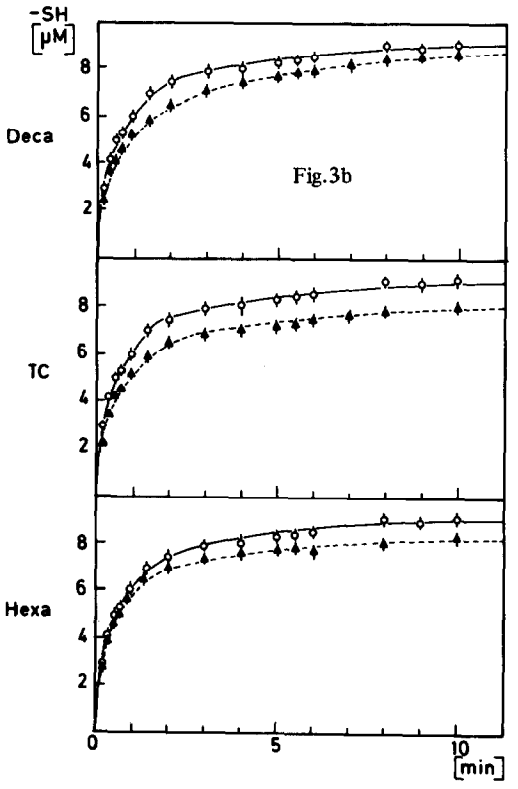


Fig.3b. Effects of 50 μM decamethonium (deca), d-tubocurarine (TC) and hexamethonium (hexa) on the reactivity of -SH of AChR-rich membranes solubilized with Triton X-100. For experimental details refer to legend to fig.3a. (○) Controls. (▲) After 40 min incubation with effectors.

[18] and therefore can be used as 'reporter groups' for structural transitions of the protein. In addition to their reactivity they are useful for the covalent attachment of a fluorescent probe.

Katz and Thesleff were the first to suggest that pharmacological desensitization is based on a decrease of ion-permeability of the membrane caused by receptors in a high affinity state for agonists [20]. Our experiments confirm the view [9,10] that this phenomenon may indeed be an in vitro model for the investigation of functional states of the acetylcholine receptor.

Figure 4 shows the decrease of the number of

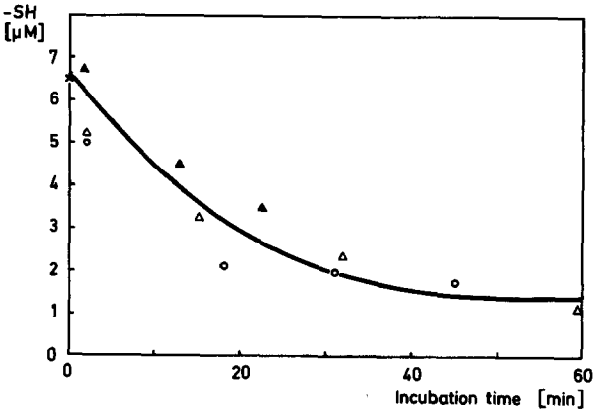
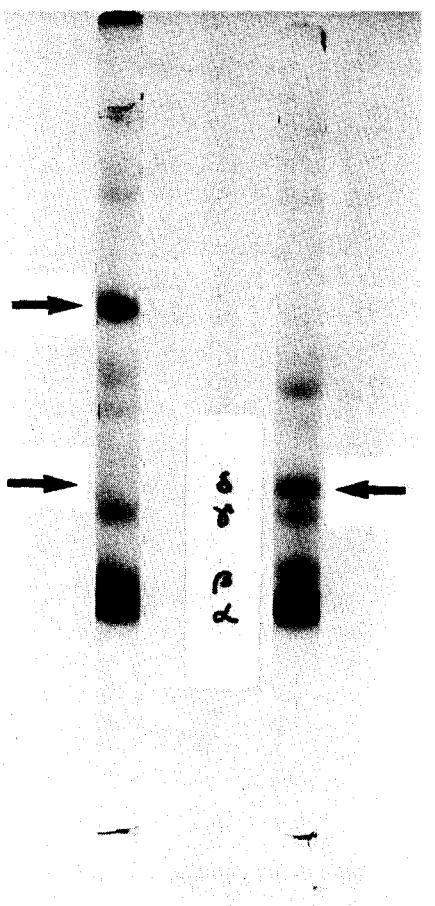


Fig.4. Effect of Triton X-100 on the reactivity of -SH groups of AChR-rich membranes. For general experimental details refer to legend to fig.1. 1 mM DTNB solution in 0.1 M phosphate buffer, pH 7.0 was shot against a membrane suspension (0.07 mg/ml protein, initial concentration) in 2% w/v Triton X-100 in 0.1 M phosphate buffer, pH 8.0. -SH Groups were determined at 25°C after different times of incubation with Triton X-100. The different symbols correspond to different experiments with the same AChR-rich membrane preparation.



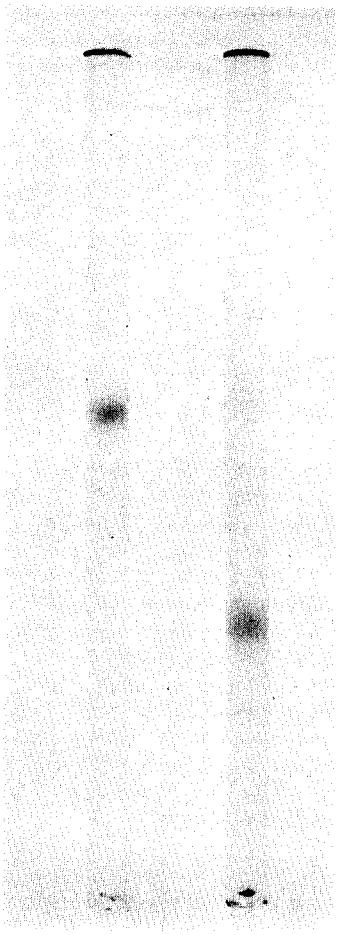


Fig.5. SDS–polyacrylamide gel electrophoresis of AChR-rich membrane with and without reduction by dithiothreitol. (a) Electrophoresis with 5% polyacrylamide gel was performed according to [21], with (right-gel) and without (left-gel) preincubation with dithiothreitol. (b) Re-electrophoresis of the protein (indicated by arrow) extracted from a gel as in (a), with (right-gel) and without (left-gel) preincubation with dithiothreitol.

-SH groups which can be reacted with Ellman's reagent after solubilization of the membrane fragments with Triton X-100. With sodium dodecyl sulfate this effect is not observed. This may indicate that Triton X-100 contains some oxidizing component. Different states of affinity have been described for the acetylcholine receptor in different detergents [8] and no cooperativity of ligand binding has been observed after solubilization of the receptor. Perhaps these observations are in part explained by the effect described in fig.4.

Sodium dodecyl sulfate does not influence the number of -SH groups to be titrated with Ellman's reagent. With this in mind one should interpret the result shown in fig.5: omitting dithiothreitol from the sample prepared for SDS–polyacrylamide gel electrophoresis, the δ-band (mol. wt 68 000) is not present in the Coomassie Blue-stained gel. Instead a new band corresponding to mol. wt 140 000 appears (fig.5a). We extracted the protein from this band and re-electrophoresed it (fig.5b): after reduction with dithiothreitol it migrated as a single band corresponding to mol. wt 68 000. This demonstrates that the new band in fig.5a arises from a dimer of the δ-poly-

peptide chain, cross-linked by a –S–S– bridge. Since in sodium dodecyl sulfate no rapid oxidation of -SH groups has been observed we conclude that this –S–S– bridge is present already in the membrane bound receptor.

Acknowledgements

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