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CONSERVATION OF THE KINETIC AND ALLOSTERIC PROPERTIES OF THE ACETYLCHOLINE RECEPTOR IN ITS Na CHOLATE SOLUBLE 9 S FORM : EFFECT OF LIPIDS

by

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SUMMARY

The rapid kinetics of interaction of the fluorescent cholinergic agonist Dns-C_6-Cho with the acetylcholine receptor protein (AChR) were followed by stopped-flow measurements. Dissolution of AChR rich membrane fragments from Torpedo marmorata by Na cholate and subsequent dilution in the absence of lipids resulted in the stabilization of the AChR in a state which exhibited a low affinity for Dns-C_6-Cho and could no longer be interconverted to the high affinity state in the presence of agonist. On the other hand, when the concentration of lipids was kept elevated in the presence of Na cholate, a soluble, 9S, low affinity form of the AChR was obtained which showed most of the characteristic properties of the membrane-bound AChR : in particular, the slow interconversion to the high affinity state and the effect of non-competitive blockers on this transition. After elimination of the detergent, this form lead systematically to successful reconstitution.

INTRODUCTION

Several experimental conditions commonly used for the solubilization and purification of the acetylcholine receptor (AChR) result in the perturbation or the loss of the characteristic kinetic and allosteric properties observed in its native membrane-bound state. For instance, after solubilization of Torpedo membranes with anionic detergents like Na cholate (1-4) or affinity chromatography in the presence of non-ionic detergents (4-9) (but in some cases solubilization alone with these detergents has similar effects (5, 9-11)), the AChR is found in a low affinity state which cannot be interconverted to a high affinity state in the presence of agonists

ABBREVIATIONS : AChR, acetylcholine receptor; Dns-C_6-Cho, {1-(5-dimethylamino-naphthalene) sulfonamido} n-hexanoic acid β-(N-trimethylammonium bromide) ethyl ester.

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and is no longer sensitive to non-competitive blockers like the local anesthetics (1-4). On the other hand, high affinity receptor sites can be obtained in the presence of non-ionic detergents (4, 5, 7-9, 11) under conditions of limited delipidation of the receptor protein (9), but in this case the binding measurements were performed under equilibrium conditions and recent rapid kinetic experiments (Heidmann, unpublished) suggest that these sites were already present in the high affinity "desensitized" state prior to the addition of the agonist. The use of the fluorescent agonist Dns-C_6-Cho (2, 12-16) combined with rapid mixing techniques makes possible the analysis of the binding properties of the AChR both in its membrane-bound (2, 13-16) and detergent-soluble forms (2); in this report, it is demonstrated that in the soluble Na cholate extract the characteristic kinetic and allosteric properties of the AChR protein can be conserved as long as lipids are present at a sufficiently high concentration. Experimental conditions can therefore be defined for the purification of the AChR in a functional state and its subsequent reintegration into artificial lipid membranes under the same state.

MATERIAL AND METHODS

Purified alkaline-treated membranes from Torpedo marmorata and dissolution by Na cholate : The AChR-rich membrane fragments were purified from freshly dissected electric tissue from T. marmorata as previously described (17) and were depleted from the 43 K protein by pH 11 treatment as described in (18) with the modification in (16). The alkaline-treated membranes were dissolved as described in (16), in 3 % (W/V) Na cholate and 0.6 % 2-mercaptoethanol; after shaking for 45 sec at room temperature, the mixture was diluted 3 times with buffer I (100 mM NaCl, 10 mM Na-phosphate buffer pH 7.4) to prevent prolonged exposure to high Na cholate concentration, and centrifuged at 100 000 x g for 20 min in a Beckman 65 rotor to pellet the insoluble material. The soluble AChR protein was kept as a concentrated solution (the "concentrated soluble extract", approx. 20 µM α-toxin binding sites and 4 mg protein/ml) in the presence of the endogenous lipids.

Assays : the AChR sites were assayed with α{^{125}I} bungarotoxin (50-150 Ci/mmole) by column filtration as described in (17). Proteins were assayed by the method of Lowry et al. (19) using bovine serum albumine as the standard.

Chemicals : Dns-C_6-Cho was a gift of G. Waksman, M.C. Fournié-Zaluski and B. Roques who synthesized it (12); {^{125}I}-labelled bungarotoxin was from NEN, Na cholate (analytical grade) from Merck and asolectin from Associated Concentrates (USA).

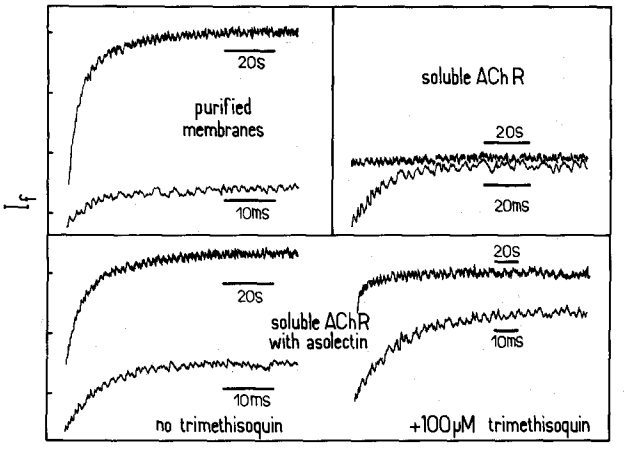

Fig. 1: Single-shot traces of stopped flow experiments; change of fluorescence intensity after rapid mixing of a solution of Dns-C_6Cho with purified alkaline-treated membranes (top left) and with the soluble AChR diluted in 1% Na cholate (top right); protective effect of added lipids (bottom), in the absence (bottom left) or presence (bottom right) of trimethisoquin.
λ_{ex} = 290 nm, λ_{em} > 540 nm; control: 1:1 mixing of a suspension of purified alkaline-treated membranes in buffer I (0.4 μM α-toxin binding sites, 0.11 mg protein/ml), with a solution of Dns-C_6-Cho (3 μM) in the same medium (top left). The 1% Na cholate concentrated soluble extract was diluted 50 fold (0.4 μM final α-toxin binding sites concentration, 0.09 mg protein/ml) in buffer I with 1% Na cholate supplemented (bottom) or not (top right) with 0.2% asolectin (see Methods); 1:1 mixing with solutions of Dns-C_6-Cho (3 μM) in buffer I, 1% Na cholate, in the presence (bottom) or absence (top right) of 0.2% (w/v) asolectin; trimethisoquin, when added (bottom right), was preincubated with the receptor solution at a concentration of 100 μM and was present at the same concentration in the Dns-C_6-Cho solution. Rapid relaxation processes are represented on expanded time scales.

RESULTS

Binding experiments were carried out after 20-50 fold dilution of the concentrated soluble extract in buffer I with 1 % Na cholate, by rapidly mixing the diluted soluble extract in the stopped-flow apparatus with a solution of the fluorescent agonist Dns-C_6-Cho in the same medium.

Under these conditions, as previously reported (2), the rapid kinetics of Dns-C_6-Cho binding differed markedly from those of the membrane-bound protein under similar conditions of site and ligand concentration (Fig. 1). In the presence of 1.5μM Dns-C_6-Cho, a single relaxation process was observed in the millisecond time range (Fig. 1) and analysis of the total signal amplitude after complete equilibration as a function of Dns-C_6-Cho concen-

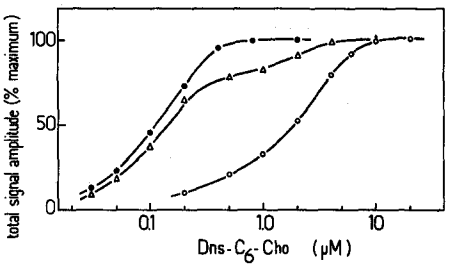

Fig. 2: Amplitude of the fluorescence intensity increase monitored after rapid mixing of purified alkaline-treated membranes (● - ●), 1% Na cholate soluble AChR in the presence (Δ - Δ) or absence (O - O) of asolectin, with increasing concentrations of Dns-C_6-Cho. Same experimental conditions as in Fig. 1.

tration (Fig. 2) revealed that the AChR were blocked in a low affinity state (approx. 2 μM) and could not undergo the transition in the second time range to the high affinity state.

Different results were obtained when the 1 % Na cholate concentrated soluble extract was diluted to the same final concentration in α-toxin sites as above, but in a 1% Na cholate saline solution supplemented with lipids (asolectin, see Materials). The stopped-flow traces obtained in these conditions markedly differed from those obtained with the diluted soluble AChR in the absence of added lipids and resembled those given with the membrane-bound AChR. The total amplitude of the fluorescence signal was close to that recorded with the membrane-bound AChR, and as expected for an increase of the affinity of the AChR site from a low affinity (2 μM, see Fig. 2) to a high affinity state, was larger than that recorded, under identical concentration conditions (1.5 μM Dns-C_6-Cho), with the soluble AChR in the absence of added lipids. Moreover, the signal exhibited the multiple relaxation processes typical of the membrane-bound AChR (see Fig. 1). A plot of the amplitude of the fluorescence signal as a function of Dns-C_6-Cho concentration (see Fig. 2) confirmed these observations: the curve obtained was close to that obtained with the membrane-bound AChR and revealed high affinity binding sites; a deviation, however, was observed in the high concentration range, which could simply be accounted for by assuming that a small but not negligible fraction

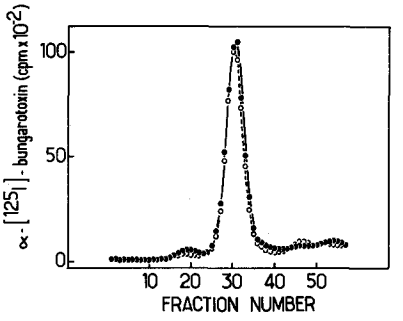

Fig. 3: Sucrose gradient ultracentrifugation of the soluble AChR supplemented with exogenous lipids (● - ●); control in the absence of added lipids (O - O).
The concentrated soluble extract (see Methods) labelled with α-{^{125}I}-bungarotoxin was diluted 30 fold in buffer I supplemented with 1% Na cholate and 0.2 % (w/v) asolectin, layered on top of a 5-20 % (W/W) sucrose gradient in buffer I, 1 % Na cholate, 1 °/₀₀ 2-mercaptoethanol and 0.2 % asolectin, and centrifuged for 14 hours at 34 Krpm in a Beckman SW 41 rotor (● - ●); control in the absence of asolectin both in the sample and the gradient, under similar conditions as above (O - O).

(approx. 30%) of the AChR was blocked in a low affinity state as observed with the soluble AChR in the absence of added lipids. Finally, the "allosteric" effect of non-competitive blockers such as the local anesthetics on the binding properties of the AChR site was still observed: preincubation of the receptor solution with the local anesthetic trimethisoquin prior to mixing with Dns-C_6-Cho resulted in an increase of the relative amplitude of the rapid relaxation signal (see Fig. 1), as observed with the membrane-bound AChR (2, 15, 16) (the total signal amplitude was also reduced, but as a consequence of spectroscopic absorption by trimethisoquin molecules). At variance with what was observed with the membrane-bound AChR, however, the effect developed at significantly higher (20 fold) trimethisoquin concentration.

Finally, it was ascertained that the addition of exogenous lipids did not alter the state of aggregation of the protein. Upon centrifugation of the soluble extract in 1 % Na cholate and 0.2 % asolectin on a 5-20 % sucrose gradient in the same medium (see Fig. 3), more than 90 % of the AChR protein migrated with the typical 9 S sedimentation coefficient, as in the control experiment carried out in the absence of added lipids. Moreover, the traces obtained with the 9 S fractions collected from the gradient were identical to those obtained with the material layered on top of the gradient.

DISCUSSION

These observations demonstrate that the characteristic binding and allosteric properties of the AChR can be conserved in its 9 S Na cholate soluble form. They do not require its integration into a native membrane environment, but rather appear strictly determined by the protein structure. Nevertheless, under alternative conditions, Na cholate may exert a deleterious effect resulting in an irreversible stabilization of the receptor protein in a low affinity state. Added lipids, but also endogenous ones if their concentration remain elevated, (data not shown), may protect against this deleterious effect of Na cholate. In this respect, specific interactions between lipid molecules and the membrane-bound protein (21) and even between the purified protein and lipid monolayers (22, 23) have been demonstrated, and it has been reported that the high affinity binding properties of the receptor protein in neutral detergents, as determined by equilibrium dialysis, were lost upon delipidation (9). Finally, recent experiments have shown (29) that the loss of the characteristic binding properties of the receptor accounts for the lack of reproductibility of the reconstitution experiments carried out with soluble and purified receptor protein in the absence of lipids (24, see 25). On the other hand, the maintainance of a high lipid concentration during the solubilization preserves these properties and leads to successful and reproducible reconstitution after eliminating the detergent (16, 26-29).

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