RECOVERY OF ALLOSTERIC INTERACTIONS BETWEEN A FLUORESCENT CHOLINERGIC AGONIST AND LOCAL ANESTHETICS AFTER REMOVAL OF THE DETERGENT FROM CHOLATE-SOLUBILIZED MEMBRANE FRAGMENTS RICH IN ACETYLCHOLINE RECEPTOR

Thierry HEIDMANN, André SOBEL and Jean-Pierre CHANGEUX

Neurobiologie Moléculaire et Laboratoire Associé Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, 75015 Paris, France

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1. Introduction

The macromolecule which, in the subsynaptic membrane, carries the physiological receptor site for acetylcholine (ACh), the acetylcholine receptor protein (AChR), is a rather well characterized macromolecule (reviewed [1,2]). Yet, the identity of the ion gate, or ionophore [3], which is under its command remains to be established without ambiguity. In particular, is the ACh-ionophore part of the purified receptor protein or, on the opposite, an entirely distinct macromolecule, highly coupled with the receptor protein in the subsynaptic membrane in situ? One evident approach to answer this question is to try to 'reconstitute' the permeability response to ACh starting from well defined components of the subsynaptic membrane dispersed into solution (reviewed [4]). The experiment appears feasible [5–7] but its rather low reproducibility suggests that some basic elements of the system yet are not under control. Another approach is based on the labeling of the ACh-ionophore by characteristic ligands followed by the identification and characterization of the macromolecule to which they bind. Electrophysiological evidence indicates that the amine local anesthetics, like prilocaine or quotane (refs. in [1]), and the frog toxin, histrionicotoxin [8–10], interact with the ACh-ionophore and are potential labels of this structural entity.

Interestingly, these compounds behave, in vitro, as allosteric effectors of the AChR-site in AChR-rich membrane fragments purified from *Torpedo* electric organ [11,12]. Moreover, this interaction is lost when the membrane fragments are solubilized by detergents [11,13,14]. Finally, a protein which binds histrionicotoxin and the fluorescent local anesthetic, quinacrine, has been isolated from the same fragments [15]. In many respects, it differs from the AChR-protein. In particular, by electrophoresis in denaturing polyacrylamide gels it gives a single band of app. mol. wt 43 000 distinct from the mol. wt 40 000 band given by the purified AChR-protein.

In order to understand the functional significance of this '43 k protein', we attempted, first, to recover the allosteric interaction between AChR-site and local anesthetic binding site starting from a crude detergent soluble extract of AChR-rich membranes containing both the AChR and the 43 k protein in a soluble form. A convenient method to follow the recovery of this interaction is to record the effect of local anesthetics on the fast kinetic of interaction of a fluorescent cholinergic agonist C_5DAChol [16–19] with the AChR-rich membrane fragments. In this letter we report that, when the concentration of detergent is lowered by dilution [13], the effect of local anesthetics on the kinetics of interaction of C_5DAChol with the AChR-site can, indeed, be 'reconstituted'.

Abbreviations: C_5DAChol, { 1-(5-dimethylaminonaphthalene) sulfonamido } *n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester; Trimethisoquin, 1-(β-trimethylaminoethoxy) 3-*n*-butylisoquinoline hydrochloride

2. Materials and methods

2.1. *Preparation of AChR-rich membrane fragments*

The AChR-rich membrane fragments were prepared by the method in [20] from freshly dissected electric tissue from *Torpedo marmorata*, and stored at −195°C in bidistilled water containing 0.1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) and 0.02% NaN_3.

The concentration of AChR-sites was measured with *Naja nigricollis* α-[^{3}H]toxin (12.4 Ci/mmol) by ultracentrifugation [21]. Proteins was estimated by the method in [22] using bovine serum albumin as standard. The specific activity of the preparations used ranged from 1500–3000 nmol α-toxin binding sites/g protein.

2.2. *Solubilization of the membrane fragments by Na-cholate*

The membrane fragments were solubilized by the method in [20] for the purification of the ACh receptor, with the following modifications: 0.5 ml concentrated suspension of membrane fragments in bidistilled water (30–50 μM α-toxin binding sites, 20 mg protein/ml) supplemented with 30 mM 2-mercaptoethanol was mixed with an equal volume of a solution of 10% (w/v) Na cholate, 200 mM NaCl, 30 mM 2-mercaptoethanol and 10 mM Na phosphate buffer (pH 7.0) in bidistilled water and stirred for 30–45 s; the mixture was then diluted 5-fold into a modified *Torpedo* physiological saline solution (250 mM NaCl, 5 mM KCl, 5 mM Na phosphate buffer (pH 7.0)) to a final cholate concentration of 1%. The solution (5 ml) was then centrifuged in a 10 ml tube at 4°C for 30 min in a Beckman rotor 40 at 33 000 rev./min (~100 000 × *g*) and the supernatant collected.

2.3. *Stopped-flow experiments*

Rapid kinetic experiments were carried out in a Gibson-Durrum stopped-flow rapid mixing apparatus equipped for fluorescence detection. Fluorescence was excited at 290 nm using a 450 W Osram Xenon lamp and a grating monochromator (Jobin Yvon HRS 2); 90° fluorescence in the observation cell (quartz tube 2 mm diam. and 18 mm length) was monitored with an Hamamatsu R 376 photomultiplier using a high pass filter (540 nm cut). Single shot fluorescence signals were digitally stored in a Tracor NS 570 (12 bits 1024 points) and plotted with a *X*–*Y* recorder. The traces were analysed with a *X*–*Y* Numonics graphic analyser on line with a Wang 2200 computer to which were given from 20–30 points/curve; the experimental traces were fitted using a non-linear iterative regression program (least square fit criterion) by mono- or multi-exponentials, and both amplitudes and rate constants determined.

2.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as in [20] on 1.1 mm thick, 10% acrylamide slab gels by the method in [23] as modified [24]. Destained gels were scanned with a Vernon gel scanner.

2.5. *Chemicals*

C_5DAChol was a gift of Dr G. Waksman, M. C. Fournié-Zaluski and B. Roques who synthesized it; the details of the procedure for synthesis are given in [16].

The purified α-toxin from *N. nigricollis* was a gift of Dr P. Boquet and was tritiated by Drs A. Menez, J. Morgat and P. Fromageot; prilocaine hydrochloride and trimethisoquin (1-(β-trimethylaminoethoxy) 3-*n*-butylisoquinoline hydrochloride) were a gift from the Laboratoire Roger Bellon (France); Na cholate (analytical grade) was from Merck.

3. Results

3.1. *Solubilization of the AChR-rich membranes by Na cholate*

AChR-rich membrane fragments (spec. act. 1500–3000 nmol α-toxin sites/g protein) were first solubilized in 5% Na cholate, then diluted to 1% cholate, and finally centrifuged at 100 000 × *g* for 30 min yielding the 1% cholate-soluble extract (see section 2). Under these conditions, ~90% of the α-[^{3}H]toxin binding sites remain in the supernatant. The initial crude extract and the supernatant were submitted to unidimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate following the procedure in [24] (see section 2). As reported [15,20], two major protein bands are observed in the uncentrifuged crude extract of the AChR-rich membranes (fig.1). Their app. mol. wt are

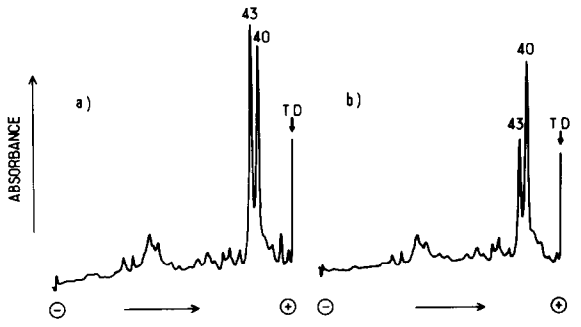

Fig.1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of (a) AChR-rich membrane fragments solubilized in cholate and (b) the same extract after 30 min centrifugation at 100 000 × *g* (cholate-soluble extract) (see section 2). Densitometric scan of the gel stained with Coomassie brilliant blue. T.D., tracking dye; the numbers above the bands indicate the apparent molecular weights (× 10^{-3}) of the corresponding polypeptide chains. Aliquots, 15 μl, of solubilized membrane fragments rich in AChR (~4 μM α-toxin binding sites, 2.7 mg protein/ml, 1% cholate) were pipetted before (a) and after (b) centrifugation at 100 000 × *g*, incubated for 1 min at 100°C with equal volumes of a solution of 2% sodium dodecyl sulfate, 0.065 M Tris–HCl (pH 6.8), 10% glycerol and 1% 2-mercapthoethanol, and submitted to electrophoresis on a 10% acrylamide gel; the electrophoresis was carried out at a constant current of 30 mA for 3–4 h and the gel fixed in 25% isopropanol/10% acetic acid/65% water for 1 h, stained overnight with 0.25% Coomassie brilliant blue in the same medium and destained with the fixation medium.

40 000 ± 1000 and 43 000 ± 1000. Densitometric scan of the gels stained with Coomassie blue shows that these bands are present in almost equal amounts. After high speed centrifugation and with some preparations of membrane fragments most of the 43 000 mol. wt band is recovered in the pellet [15]; with the membrane preparations used in this work, ~90% of the 40 000 mol. wt and 50% of the 43 000 mol. wt bands remain in the supernatant after centrifugation.

Centrifugation of the cholate extract on a 5–20% (w/v) sucrose gradient gives a single and symmetrical peak of α-[^{3}H]toxin-binding material with a standard sedimentation coefficient of 9 S characteristic of the soluble form of the AChR protein and the 43 k protein does not comigrate with it (A. S., unpublished results).

The 1% cholate-soluble extract, therefore, contains the AChR-protein and other components of the AChR-rich membranes, such as the 43 k protein, in a dispersed and soluble form.

3.2. *Interaction of C_5DAChol with the AChR-site in the 1% cholate-soluble extract*

It was shown [17] that rapid mixing of AChR-rich membrane fragments with C_5DAChol gives rise to an increase of C_5DAChol fluorescence intensity which takes place in the millisecond to second time range under conditions of energy transfer from proteins. Three major relaxation processes are observed, a 'rapid' one assigned to the interaction of C_5DAChol with a state of the AChR with a high affinity for agonists, an 'intermediate' one accounted for by a binding of C_5DAChol to AChR sites in a state of lower affinity and a 'slow' one analysed in terms of an interconversion between these low and high affinity states of the AChR [17,18]. In the presence of local anesthetics, the amplitude of the rapid relaxation process and the rate constant of the slow relaxation process increase [17,19]: local anesthetics stabilize, in an 'allosteric' manner, the high affinity state of the AChR.

When the 1% cholate-soluble extract (~0.2 μM α-toxin binding sites) is rapidly mixed with C_5DAChol (in 1% cholate), an increase of C_5DAChol fluorescence intensity is also recorded in the milliseconds time range under conditions of energy transfer from proteins (fig.2). The signal is abolished by preincubation of the soluble extract with saturating concentrations of *N. nigricollis* α-toxin. It is therefore associated with the interaction of C_5DAChol with the AChR-site. At variance with what is observed with the native membranes, the traces can be fitted in first approximation by a single exponential. Figure 3 shows that the amplitude of the fluorescence signal increases with increasing C_5DAChol concentration up to a plateau value, and with an apparent dissociation constant equal to ~1.8 μM. A Scatchard plot of the data reveals however a minor but significant heterogeneity (see also [13]) with at least two classes of binding sites with app. K_d ~1.3 μM and ~5.3 μM. As shown in fig.4, the k_{app} of the signal increases linearly with increasing C_5DAChol concentration.

In first approximation, neglecting this heterogeneity, the data can be accounted for by a bimolecular bind-

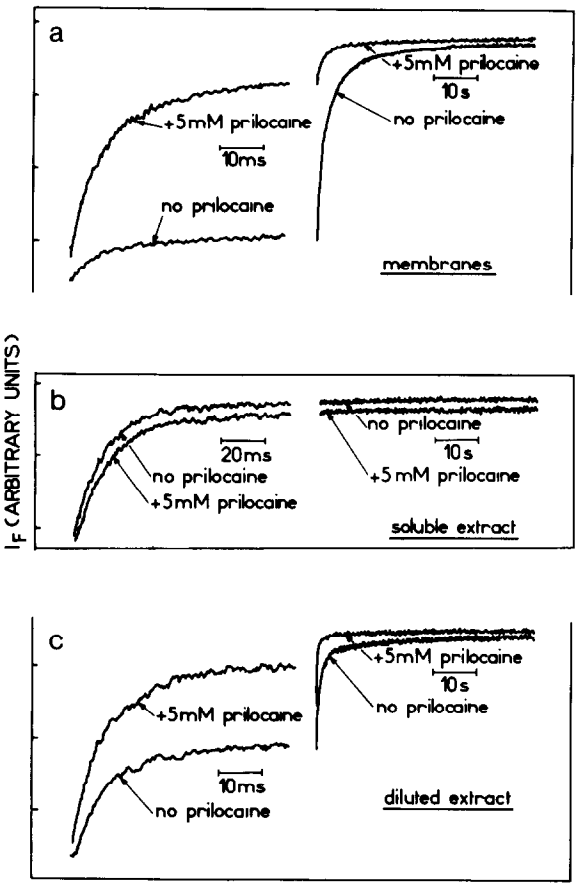

Fig.2. Single-shot traces of stopped-flow experiments: change of fluorescence intensity after rapid mixing in the presence and in the absence of 5 mM prilocaine of a solution of C_5DAChol with (a) native membrane fragments, (b) 1% cholate-soluble extract, and (c) diluted cholate extract. (a) 1:1 mixing of a solution of C_5DAChol in *Torpedo* saline solution (~0.4 μM α-toxin binding sites, 0.25 mg protein/ml) supplemented or not with 5 mM prilocaine (at least 30 min preincubation). (b) The 1% cholate-soluble extract obtained after high speed centrifugation (see section 2) was diluted 200-fold at a constant cholate concentration, in a *Torpedo* saline solution supplemented with 1% cholate (~0.4 μM α-toxin binding sites); 1:1 mixing with a solution of C_5DAChol in *Torpedo* saline solution (same concentration as in (a)) supplemented with 1% cholate was done at least 30 min later; prilocaine was added as indicated above. (c) The 1% cholate-soluble extract obtained after high speed centrifugation was diluted 200-fold in *Torpedo* saline solution (~0.4 μM α-toxin binding sites, 0.05% w/v final cholate concentration); 1:1 mixing with a solution of C_5DAChol in *Torpedo* saline solution (same concentration as in (a)) supplemented with 0.05% cholate was done at least 30 min later; prilocaine was added as above.

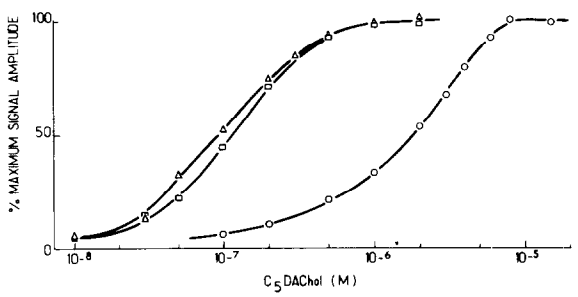

Fig.3. Amplitude of the fluorescence intensity increase monitored after rapid mixing of native membrane fragments (□–□), 1% cholate soluble (○–○) and diluted (△–△) extracts with increasing concentration of C_5DAChol. Same conditions as for fig.2.

ing reaction $R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$. Under these conditions and in the presence of an excess of ligand, $k_{app} = k_1 \{L\} + k_{-1}$ and, as observed experimentally, increases linearly with ligand concentration. The slope and intercept with the ordinate axis are $k_1 = 1.8 \times 10^7$ M^{-1} s^{-1} and $k_{-1} = 33$ s^{-1}.

With the 1% cholate-soluble extract, local anesthetics such as prilocaine or trimethisoquin no longer modify the amplitude and rate constant of the signal monitored upon rapid mixing with C_5DAChol. At

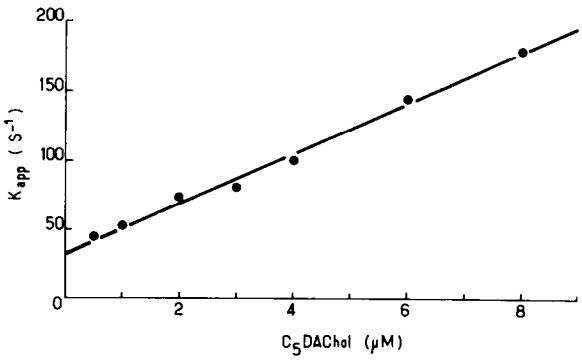

Fig.4. Fluorescence intensity increase monitored after rapid mixing of C_5DAChol with the 1% cholate-soluble extract: plot of the apparent first-order rate constant, k_{app}, as a function of the final C_5DAChol concentration. Same conditions as for fig.2b; the solid line is a least square fit of the data: intercept with the ordinate axis 33 s^{-1}, slope 1.8×10^7 M^{-1} s^{-1}.

prilocaine >10 mM, the amplitude of the fluorescence signal decreases without significant change of the app. K_d for half maximum signal amplitude; the k_{app} values are not modified, which suggests that the observed decrease in fluorescence intensity is due to a non-specific spectroscopic absorption by the local anesthetic rather than to a change in the binding properties of the AChR.

In summary, solubilization by 1% cholate causes a marked change in the binding properties of the AChR protein which differ from the high affinity state present in the native membranes. In addition the sensitivity to local anesthetics is lost.

3.3. *Quantitative analysis of the effect of cholate on the AChR-rich membrane fragments*

Na cholate acts as a dispersive agent at concentrations close to its critical micellar concentration (c.m.c. ≃0.65% w/v). At lower concentrations (i.e. ~100-fold below the c.m.c.) it blocks in vivo the response of the electroplaque to agonists in a non-competitive manner and behaves in this respect like a local anesthetic [14,25]. An important question is whether or not this last effect accounts, at least in part, for the particular properties of the receptor protein in 1% cholate. To answer this question, the effect of cholate on the AChR-rich membrane fragments was quantitatively studied as a function of cholate concentration. Membrane fragments were preincubated with cholate from 0.001–1% (w/v), and thereafter mixed in the stopped-flow apparatus with a solution of 2 μM C_5DAChol supplemented with the corresponding concentration of cholate (in order to keep the cholate concentration constant). In these conditions, the fluorescence signal obtained resembles that found with typical local anesthetics [17,19]. The amplitude of the rapid relaxation process increases up to a plateau value which represents ~65% of the total signal amplitude (fig.5) with, for this effect, an app. K_d ~0.06% w/v (~1.4 mM). At higher concentrations and upon increasing cholate concentration from 0.5–1%, a marked qualitative change of the fluorescence signal is observed. The traces which can be decomposed into three distinct processes for a concentration of cholate smaller than the c.m.c. (i.e. up to 0.5% cholate) become monophasic in the presence of 1% cholate as in the case of the soluble

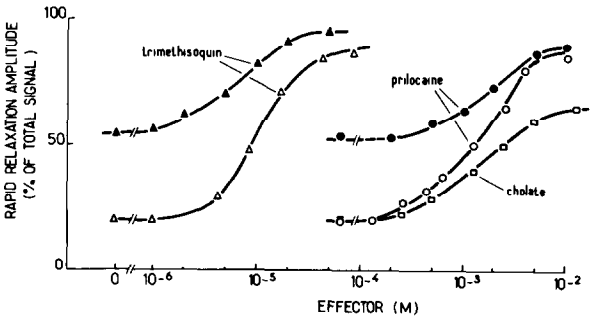

Fig.5. Effect of local anesthetics and cholate on the relative amplitude of the rapid relaxation process monitored with the native membrane fragments (open symbols) and the diluted cholate extract (filled symbols). The amplitudes of the rapid relaxation process are given as % of the total amplitude of the fluorescence signal (which is not modified by the presence of local anesthetics in the domain of concentration studied) for cholate (□–□), prilocaine, (○–○; ●–●) and trimethisoquin (△–△; ▲–▲). Same experimental conditions as for fig.2c (●–●; ▲–▲). Data from [19] (○–○; △–△). Same experimental conditions as for fig.2a (□–□).

extract in the previous experiments. Conversely, the rate constant of the monophasic signal recorded in the presence of 1% cholate becomes ~2-times slower than the rapid relaxation process monitored in the presence of 0.5% cholate and cannot be related to any of the two other (slow and intermediate) relaxation processes. In other words, when the concentration of cholate increases from 0.5–1% the kinetic and binding properties of the AChR-protein follow a discontinuous and sharp transition which cannot be assigned to a local anesthetic effect of cholate but rather to a change in the environment of the receptor protein.

3.4. *Recovery of the binding properties of the AChR-protein after removal of cholate by dilution*

In an attempt to recover the properties of the membrane-bound AChR the concentration of cholate in the soluble extract was reduced by dilution to a final concentration of 0.05% w/v, below the critical micellar concentration (0.65% w/v cholate). The diluted extract (~0.2 μM α-toxin sites, 0.05% cholate) was then rapidly mixed in the stopped-flow apparatus with various concentrations of C_5DAChol in *Torpedo* saline solution (supplemented with 0.05% cholate). Under these conditions a change of fluorescence

intensity is recorded (fig.2) which, again, is abolished by preincubation with saturating concentrations of *N. nigricollis* α-toxin. The amplitude of the fluorescence signal increases with increasing C_5DAChol concentrations (fig.3). A plot on a linear scale of the amplitude as a function of C_5DAChol concentration resembles a titration curve with a linear increase of the amplitude, up to a plateau value at conc. >0.2 μM (the concentration in α-toxin binding sites). The midpoint of the curve takes place at C_5DAChol ~0.1 μM, which is approximately one order of magnitude smaller than the corresponding value measured in the case of the 1% cholate extract but still is an overestimate of the microscopic equilibrium dissociation constant.

At variance with what is observed with the 1% cholate-soluble extract, the traces are no longer monophasic but display, for given C_5DAChol concentrations, the three relaxation processes characteristic of the native membrane fragments [17–19]. Moreover, the rate constants of the three processes and their dependence with C_5DAChol concentration appear very similar to those found with the native membranes (fig.6).

The data can be accounted for by the same two-state model as that used to fit the results given by the AChR-rich membranes [18,19]. The model postulates, in particular, the interconversion of the AChR between two conformational states with a low (~1 μM) and high (~3 nM) affinity of the ACh binding site for C_5DAChol. Only minor differences were noticed between the diluted extract and the native membranes: a slightly higher rate constant of the slow relaxation process and a larger amplitude of the rapid relaxation process. With the diluted cholate extract, this last amplitude represents as much as ~50% of the total amplitude of the fluorescence intensity change, against only 20% in the case of the native membrane fragments. In other words, the fraction of high affinity sites present spontaneously in the cholate-diluted extract is larger than in the native membranes.

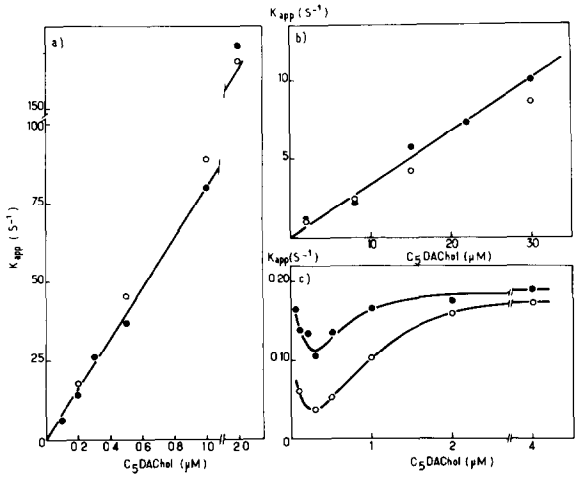

Fig.6. Plot of the k_{app} values of the rapid (a), intermediate (b) and slow (c) relaxation process as a function of C_5DAChol concentration, for the AChR-rich membrane fragments (○–○) and the diluted cholate extract (●–●). Same experimental conditions as for fig.2 (a,c).

Interestingly, dilution of the cholate extract leads to a recovery of the sensitivity to local anesthetics which, in many instances, appears similar to that of the membrane-bound AChR. In the presence of two typical local anesthetics such as prilocaine and trimethisoquin, the amplitude of the rapid relaxation process increases, without change in the total amplitude of the fluorescence signal recorded after complete equilibration (fig.5). The effect takes place in a domain of concentration similar to that observed with the native membrane fragments, with app. K_d = 1.5 mM for prilocaine and 7 μM for trimethisoquin (versus 1.5 mM and 10 μM, respectively, for native membrane fragments). Furthermore, and in agreement with what is observed with native membrane fragments, the rate constants of the intermediate and slow relaxation processes increase, whereas that of the rapid relaxation process slightly decreases. In both cases, the data can be interpreted in terms of a stabilisation of the high affinity state of the AChR by local anesthetics.

Upon removal of the detergent, the AChR therefore regains equilibrium binding properties, conformational transition kinetics and sensitivity to local anesthetics almost identical to those measured with the native membrane fragments.

Recovery after cholate dilution occurs rapidly. No difference was detected in the traces recorded 2 min (which is the shortest time needed for dilution and introduction of the solution in the stopped-flow syringes) and several hours after dilution. Time-dependent effects, however, were noticed at the level

of the solubilization process. Prolonged storage of the concentrated (5%) cholate extract leads to a decrease of the fluorescence signal monitored after dilution of the cholate extract, which cannot be accounted for by a change in the binding properties of the AChR but rather by an irreversible decrease in the number of binding sites (50% decrease after 45 min and almost 100% after 3 h). On the other hand, no significant time-dependent effect is observed upon storage in 1% or, a fortiori, 0.05% cholate.

After cholate dilution, the AChR-protein followed by α-[^{3}H]toxin binding is no longer in a soluble form and can be sedimented by centrifugation at 100 000 × *g*. Polyacrylamide gel electrophoresis of the pellet in the presence of sodium dodecyl sulfate yields both the mol. wt 40 000 and 43 000 bands. In other words, the dilution of the cholate extract which results in the recovery of the most characteristic properties of the membrane-bound AChR also causes an aggregation of the two major protein components of the AChR-rich membranes.

4. Conclusion

Upon solubilization of the AChR-rich membrane fragments by cholate a marked change in the binding properties of the AChR-protein takes place. In particular, and in agreement with previous work, the sensitivity to local anesthetics is lost. In other words, the binding sites for ACh and for local anesthetic are phenomenologically uncoupled.

At least three explanations for this effect are possible:

1. The local anesthetic binding site itself is occupied or hidden by the detergent which therefore interferes directly with the binding of local anesthetics to their proper site of action.

2. The coupling between the local anesthetic and the ACh binding sites is lost upon detergent solubilization as a consequence of a physical uncoupling of the protein units which carry the two classes of sites.

3. In the presence of detergent the AChR is 'frozen' in one (or multiple) affinity state which excludes conformational transitions between affinity states.

The first hypothesis is plausible, but appears unlikely since cholate acting as a local anesthetic should stabilize the membrane-bound AChR in a high affinity state which is clearly distinct from the low affinity state found in the cholate-soluble extract. Hypotheses 2 and 3 are not mutually exclusive and could account for the effect. Recent progress in the identification of the major polypeptide chains present in the highly purified membrane fragments suggest that the local anesthetic binding site could be carried by the 43 k protein, distinct from the 9 S soluble macromolecule identified as the AChR protein. These two proteins are still present in the 1% cholate extract, but are no longer physically coupled.

When the concentration of the detergent is lowered by dilution, the binding properties of the AChR and the sensitivity to local anesthetics become very close to those found with the native membrane fragments. Only minor differences are noticed. For instance, the fraction of AChR sites in a high affinity state before the addition of C_5DAChol, and the rate constant of the slow relaxation process are higher with the diluted cholate extract than with the native membrane fragments. At least two interpretations may account for these observations:

1. The intrinsic properties of the AChR are strictly identical before and after solubilization, but traces amount of cholate are still present after dilution and change both the equilibrium and dynamic properties of the AChR. As mentioned in section 3, cholate indeed exerts a local anesthetic action in vivo, and modifies in vitro the kinetics of the interaction of C_5DAChol with the membrane-bound AChR in the expected direction.

2. The solubilization process, the centrifugation and the final dilution alter the binding properties of the AChR as a consequence possibly of a change in the lipid environment and/or incomplete reconstitution.

In any case, the main features of the allosteric interaction between AChR-site and local anesthetic binding site can be 'reconstituted' after elimination of the detergent from a crude extract of subsynaptic membrane fragments. Under these conditions, AChR and 43 k protein aggregate into high molecular weight

particles. This method can therefore be used to test the functional significance of the 43 k protein.

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