Allosteric transitions of *Torpedo* acetylcholine receptor in lipids, detergent and amphipols: molecular interactions vs. physical constraints

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Abstract The binding of a fluorescent agonist to the acetylcholine receptor from *Torpedo* electric organ has been studied by time-resolved spectroscopy in three different environments: in native membrane fragments, in the detergent CHAPS, and after complexation by amphipathic polymers ('amphipols'). Binding kinetics was similar in the membrane and in amphipols, demonstrating that the receptor can display unaltered allosteric transitions outside its natural lipid environment. In contrast, allosteric equilibria were strongly shifted towards the desensitized state in CHAPS. Therefore, the effect of CHAPS likely results from molecular interactions rather than from the loss of bulk physical properties of the membrane environment.

Key words: Nicotinic acetylcholine receptor; Conformational transition; Membrane protein; Detergent; Amphipol

1. Introduction

Transmembrane proteins are adapted to an environment comprised of two distinct aqueous media and the highly complex membrane phase [1]. Handling them in aqueous solution requires their complexation by amphipathic molecules that screen their hydrophobic transmembrane surface from contact with water. Traditionally, this role is fulfilled by detergents. Detergents are small surfactants that cooperatively assemble at the transmembrane surface of the protein at concentrations close to their critical micellar concentration (CMC) [2]. When the concentration of free detergent is lowered below the CMC, most molecules desorb and the protein becomes insoluble. Membrane proteins therefore have to be handled in the presence of excess detergent micelles. Detergents are, by definition, endowed with dissociating properties, which frequently prove harmful: perturbation of functional properties if not downright inactivation by detergents is a recurrent concern in membrane protein biochemistry.

'Amphipols' (AP) are amphipathic polymers designed for making membrane proteins hydrophilic [3]. When added to a membrane protein in detergent solution, they form with the detergent a mixed layer at the surface of the protein [4]. Upon detergent removal, they stick to the protein [4], keeping it water-soluble [3–7]. Membrane proteins are not denatured by AP [3,5,6,8] and, usually, are more stable after complexation by AP than they are in detergent solutions [3,5]. Little is known, however, about the functionality of AP-complexed proteins. In the most detailed study to date, the function of the sarcoplasmic reticulum calcium pump was found to be reversibly inhibited by AP, raising the possibility that AP might interfere with protein conformational transitions [5].

The nicotinic acetylcholine receptor (nAChR) is the paradigm of a large family of chemically gated ion channels that play an essential role in cell communication [9]. The neuromuscular form is comprised of five homologous transmembrane subunits ($\alpha_2\beta\gamma\delta$) arranged around a central ion channel [9–11]. Agonists and competitive antagonists bind to sites located at interfaces between the extracellular domains of the α/δ and α/γ subunits [12,13]. In the absence of ligands, the membrane-bound receptor exists mainly ($\sim$85%) in a 'basal' state (B) that has a low affinity for ACh and is amenable to activation, the remnant being in a 'desensitized', high-affinity state (D). In both states, the ion channel is closed. Addition of nACh shifts the equilibrium first towards an active state (A), where the channel is open, and then towards the higher-affinity intermediate (I) and desensitized (D) states, both of them with a closed channel [11]. Allosteric ligands such as non-competitive antagonists regulate the equilibrium between basal and desensitized states [11]. Conformational transitions can be conveniently monitored in vitro by following the kinetics of binding of a fluorescent analog of ACh, dansyl-C_6-choline (DnsCho) [14,15].

It has long been recognized that the composition of the lipid environment of the AChR influences its activity (see e.g. [16], and references therein), and that its conformational

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equilibria can be perturbed by addition to the membrane of foreign molecules such as local anesthetics, detergents or fatty acids [17,18], as well as by solubilization with detergents [19,20]. It has remained difficult to sort out, however, to which extent these effects result from an alteration of the physical properties of the environment (see e.g. [21,22]) or from direct molecular interactions (see e.g. [16,23]). In the present work, we have re-examined this question by taking advantage of the ability of amphipols to keep membrane proteins soluble in the absence of detergent micelles.

2. Materials and methods

2.1. Materials

Except where otherwise indicated, sources of materials were as indicated in [15]. *Torpedo marmorata* was obtained live from the Laboratoires de Biologie Marine in Arcachon or Roscoff, France. CHAPS and proadifen were from Sigma, α-bungarotoxin (α-Bgt) from Molecular Probes and ^{125}I-α-Bgt from Amersham. Thin-layer chromatography (TLC) pre-coated aluminum sheets (Silicagel 60 Kieselguhr F254, 0.2 mm thick) were from Merck. Amphipol A8-35 was synthesized as described in [3] and [^{14}C]A8-35 (specific activity, 9×10^8 cpm g^{-1}) as described in [4]. 'Phosphate buffer' and 'Tris buffer' contained 5 mM Na_2PO_4 (resp. Tris/HCl), 1 mM EDTA, 100 mM NaCl, pH 7.2; 'CHAPS buffer' is phosphate buffer supplemented with 6.5 mM CHAPS.

2.2. Preparation of nAChR

Receptor-rich membranes from *T. marmorata* electric organ [24] were treated at pH 11 in order to remove extrinsic proteins [25]. Alkaline-treated membranes were solubilized by the addition of CHAPS buffer (6.5 mM final concentration) [15] to reach a final protein concentration of 1 g l^{-1}. The samples were centrifuged 45 min at $18\,000\times g$ in the 45Ti rotor of a L8 centrifuge (Beckman) and the supernatant concentrated to 15–20 g l^{-1} using Centricon 100 devices (Amicon; cut-off 100 kDa). This step ought to eliminate ~95% of free micelles.

2.3. Trapping with amphipols

In most experiments, CHAPS-solubilized nAChR (diluted to 1.4–5.8 g l^{-1} with CHAPS buffer) was supplemented with AP to reach nAChR/A8-35/CHAPS weight ratios of 1:3:0.7–3. After incubation for 20 min at 4°C, samples were diluted 10× with detergent-free phosphate buffer. The CHAPS concentration thereby fell ~7× below its CMC (~4 mM; see [26]). Samples were centrifuged 30 min at $18\,000\times g$ as described above. The yield of protein in the supernatant was 87 ± 10%.

2.4. Protein and lipid analysis

Protein concentrations were determined either as described in [27], with bovine serum albumin as a standard, or, for purified receptor, from the optical density at 280 nm, using $\varepsilon_{280} = 1.2$ g^{-1} l cm^{-1} [15]. DnsCho binding site concentrations were determined by steady-state fluorescence measurements [15].

Lipids were extracted as described in [28,29]. Extracts in chloroform were applied to TLC plates, which were developed with 65/25/4 (v/v/v) chloroform/methanol/water. Phospholipids were identified from their R_f, using standards and stains described in [30], and quantified by phosphorus analysis [31]. Cholesterol was identified from its R_f: the iodine-stained spot at the migration front was scratched from the plates, eluted in chloroform/methanol 1/1 (v/v), applied onto a second plate, and eluted along with a cholesterol standard using petroleum ether (bp 40–65)/ethyl ether/glacial acetic acid 70/30/1 (v/v/v) as a solvent.

2.5. Rate zonal centrifugation

250 μl samples were centrifuged for 17 h at $160\,000\times g$ at 4°C on 11 ml 5–20% (w/w) continuous sucrose gradients, containing or not 6.5 mM CHAPS, in the SW41 rotor of an L8 ultracentrifuge (Beckman), as described in [32]. The distribution of nAChR subunits was determined by analyzing aliquots of gradient fractions by SDS–PAGE on 10% polyacrylamide gels, which were silver-stained as described in [33].

Quantitative analysis of nAChR distribution was performed using samples preincubated for 5 h at 4°C in CHAPS buffer with a 12× molar excess of bungarotoxin (^{125}I-α-Bgt isotopically diluted with unlabeled α-Bgt). Some of the samples were then supplemented with A8-35 at a 1:80:40 AChR/A8-35/CHAPS weight ratio, incubated for 15 min at 4°C and diluted below the CMC of CHAPS. Sucrose gradient fractionation was carried out as described above. Fractions were counted in an LKB 1275 MiniGamma system. Because of the low specific activity of [^{14}C]A8-35, double-labeling was impractical, and the mass ratio of AP to protein in nAChR/A8-35 particles was estimated by combining data from parallel experiments: CHAPS-solubilized nAChR was reacted with unlabeled α-Bgt and trapped with [^{14}C]A8-35, and the resulting complexes fractionated as described above. 50 μl aliquots of each gradient fraction were supplemented with 5 ml Aqualuma Plus counting medium (Lumac LSC) and counted in a Beckman liquid scintillation system LS 1801. Control experiments included determining the distribution of free ^{125}I-α-Bgt and free [^{14}C]A8-35 in identical gradients run in parallel. Sedimentation coefficients in water at 20°C ($s_{20,w}$) were determined from these data using abacuses calculated by interpolation from published tables [34], assuming the partial specific volume of the particles to be 0.75 cm^3 g^{-1}. This value, calculated for pure nAChR [10], is underestimated given the presence of bound lipids, CHAPS and/or AP. This entails a slight underestimate of $s_{20,w}$.

For lipid analysis, unbound lipids trapped in protein-free surfactant micelles were separated from the nAChR dimer by sucrose gradient sedimentation. The heavy form of the receptor was collected and transferred to Tris buffer by five rounds of 5× concentration/dilution cycles using Centricon 10 devices.

2.6. Analysis of nAChR allosteric transitions

The kinetics of binding of DnsCho (2 μM) to the nAChR (0.4 g l^{-1} total protein) was followed at 20°C using a stopped-flow instrument equipped with fluorescence detection (Biologic), as described in [14,15,35]. Samples were excited at 296 ± 4 nm and light collected above 420 nm using a high-pass filter [15]. A 0.5 ms sampling period was used for the first second of detection and a 200 ms one for the rest of the kinetics. The time scale was corrected for the dead time of the instrument (3.1 ms). Binding kinetics was followed in the absence or presence of two antagonists, a competitive one, α-Bgt (1 μM), and a non-competitive, desensitizing one, proadifen (30 μM), after 1 h preincubation at room temperature. They were analyzed as the sum of three exponentials [14,35]:

$$F^{296} = (at + b) + \sum_{i=1}^{3} m_i e^{-k_i t}$$

where F^{296} is the fluorescence intensity detected upon excitation at 296 nm, k_i and m_i are the time constant and the amplitude of exponential i, respectively, and a and b define the straight line to which the kinetics tends. The quality of the fit was determined from the non-normalized deviation between experiment and theory at each time point:

$$\mathrm{Dev}(i) = F^{296}_{\mathrm{calc}(i)} - F^{296}_{\mathrm{exp}(i)}$$

Attempts to fit the data with four exponentials led either to no convergence or to degenerate solutions without any improvement of the average weighted residual.

3. Results

3.1. nAChR dispersity

Three kinds of preparations were analyzed by sedimentation on sucrose gradients, namely ^{125}I-Bgt-labeled nAChR in 6.5 mM CHAPS (a) and the same preparation diluted below the CMC of CHAPS following (b) or not (c) addition of AP A8-35. In case a, gradients contained 6.5 mM CHAPS; in cases b and c, they contained neither detergent nor AP. Gradient fractions were analyzed by SDS–PAGE and γ counting. When CHAPS was diluted below its CMC in the absence of AP (case c), most of the nAChR aggregated and formed a pellet at the bottom of the tube (data not shown). The absence

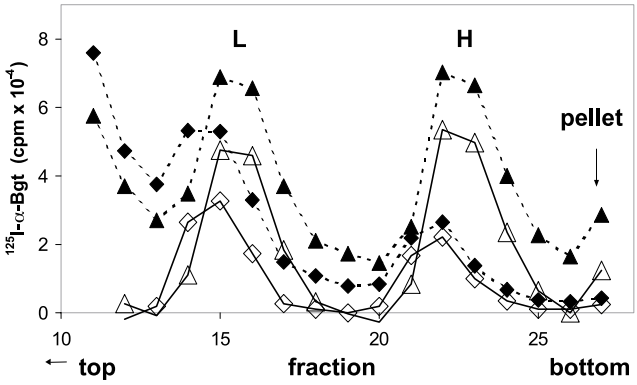

Fig. 1. Migration of CHAPS-solubilized and AP-trapped nAChR during centrifugation on sucrose gradients. CHAPS-solubilized nAChR was labeled with ^{125}I-α-Bgt. An aliquot (▲) was fractionated by ultracentrifugation on a 5–20% sucrose gradient in CHAPS buffer (see Section 2). A second aliquot (◆) was supplemented with AP A8-35, diluted 6× (i.e. ∼4× below the CMC of CHAPS) with phosphate buffer, and centrifuged on a gradient containing neither CHAPS nor AP. Solid symbols and dashed lines refer to rough data, empty symbols and solid lines to the profile obtained following subtraction of the background of free ^{125}I-α-Bgt, estimated from parallel experiments.

of radioactivity in the pellet indicated that the aggregated receptor had denatured, releasing the radioactive toxin (data not shown). Under the other two conditions, no or very little pellet was present, the nAChR migrating as a light and a heavy form (Fig. 1). Each form comprised the four receptor subunits, indicating that both CHAPS and AP had preserved the quaternary structure of the $\alpha_2\beta\gamma\delta$ oligomer (data not shown).

Both receptor forms were observed to migrate slightly more slowly once trapped by A8-35 than they did in the presence of CHAPS: $s_{20,w}$ values were 9.7 ± 0.3 s in AP vs. 10.3 ± 0.3 s in CHAPS for the light form, and 14.7 ± 0.3 s in AP vs. 15.4 ± 0.3 s in CHAPS for the heavy one. These values are close to those reported for the sedimentation coefficients of the monomeric and dimeric forms of the nAChR (8.7–9.5 s and 12.5–14.4 s, respectively, depending on the nature of the detergent; see [10,26,36], and references therein).

3.2. Specific activity

The specific activity of the samples was estimated from steady-state fluorescence measurements using DnsCho [15,37,38]. nAChR in crude membranes had a specific activity of 1.95 ± 0.25 nmol of agonist binding sites per mg of total protein (i.e. ∼30% nAChR in mass). This rose to 6.0 ± 1.4 (∼90% in mass) after alkaline treatment and solubilization by CHAPS, in keeping with the prominence of the bands corresponding to the nAChR subunits as seen by SDS–PAGE. A8-35-trapped nAChR had a specific activity of 5.3 ± 0.8 (∼80% in mass), which is not significantly different from that in CHAPS. Most if not all of the nAChR, therefore, remained able to bind agonists following trapping with AP.

3.3. Amphipol/nAChR mass ratio in the complexes

AP binding to the nAChR was examined by sucrose gradient fractionation of preparations trapped with [^{14}C]A8-35 [4]. The distribution comprised two peaks, with sedimentation coefficients corresponding to those of nAChR light and heavy forms, superimposed on a background of free AP (data not shown). The amount of AP bound to the heavy form was 0.28 ± 0.03 g/g. That bound to the light form was more difficult to establish because of the higher background of free AP, but did not appear significantly different. This ratio is comparable to that found (using a slightly different AP) for another protein of comparable transmembrane size, the cytochrome $b_6 f$ dimer [4,8].

3.4. Lipid content

TLC revealed the presence in preparations of CHAPS-solubilized and AP-trapped nAChR of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and cholesterol. This composition is similar to that of *T. marmorata* receptor-rich membranes [30]. Phosphorus analysis yielded comparable results for complexes with either CHAPS or A8-35, namely 71 ± 5 and 88 ± 6 phospholipids per nAChR dimer, respectively, suggesting that all of the lipids associated with the protein in CHAPS solution remain bound to it upon transfer to AP. This is comparable to the ∼90 phospholipids per dimer that have been found to be required for keeping solubilized nAChR from inactivating [39]. The presence of ∼130 tightly associated phospholipids per dimer of CHAPS-solubilized nAChR has been reported previously [26].

3.5. Allosteric transitions

Binding of DnsCho to the ACh binding sites was studied using stopped-flow measurements. The kinetics can be fitted by three exponentials [14,15,35]: a rapid phase (ms range) reflects binding of the ligand to receptor molecules in the high-affinity D state, whereas conformational transitions from the low-affinity B state to the I and D states give rise to the slower phases (min range). Thus, the relative amplitude of the rapid phase (Table 1, parameter m_3) reflects the fraction of nAChR that pre-exists in the D state prior to the addition of DnsCho.

In native membranes, 11 ± 2% of the nAChR population pre-exists in the D state (Fig. 2 and Table 1). Upon preincubation with the non-competitive antagonist proadifen, the desensitized population rises to 85 ± 10%. These values are in good agreement with the literature [40,41]. Similar results were obtained whether the membranes had been treated at pH 11 or not (data not shown). In CHAPS solution,

Table 1
Time constants and pre-exponential coefficients describing the kinetics of binding of DnsCho to nAChR in different environments

	Native membrane	CHAPS	A8-35
m_1 (%)	43 ± 12	26 ± 6	54 ± 3
k_1 (s^{-1})	0.06 ± 0.02	0.11 ± 0.06	0.04 ± 0.01
m_2 (%)	46 ± 11	17 ± 5	32 ± 7
k_2 (s^{-1})	0.5 ± 0.3	0.6 ± 0.3	0.31 ± 0.16
m_3 (%)	11 ± 2	57 ± 9	14 ± 5
k_3 (s^{-1})	50 ± 6	58 ± 10	85 ± 19

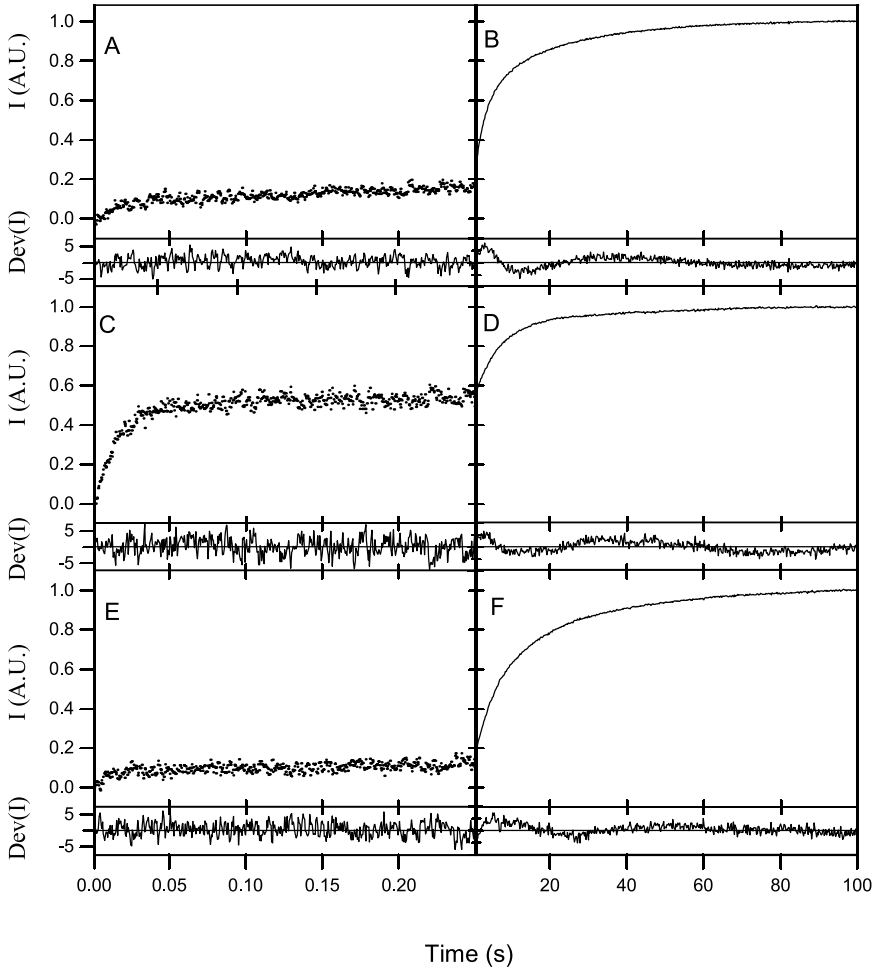

Fig. 2. Kinetics of DnsCho binding to native receptor-rich membranes (A,B), CHAPS-solubilized nAChR (C,D) and nAChR trapped by A8-35 (E,F). Kinetics is represented on two time scales, from 0 to 0.25 s for the rapid phase (A,C,E), and from 0 to 100 s for the overall process (B,D,F). Fitting parameters, averaged over 4–5 preparations, are given in Table 1. Dev(I): residual of the fit.

57 ± 9% of the nAChR population pre-existed in the high-affinity state (Fig. 2 and Table 1), rising to 80 ± 10% after preincubation with proadifen. This shift of the resting conformational equilibrium towards the desensitized state is comparable to that previously reported for nAChR solubilized in lipid/cholate [20] or lipid/Triton X-100 [35] mixtures. Following addition of A8-35 and dilution under the CMC of CHAPS, 14 ± 5% of the population pre-existed in the D state (Fig. 2 and Table 1), rising to 70 ± 9% after preincubation with proadifen. Whatever the environment (native membranes, CHAPS micelles or AP), preincubation of the nAChR with α-Bgt abolished the fluorescence transient, indicating that it does report on specific binding to the ACh binding sites. The rate of interconversion between conformational states, which is reflected in rate constants k_1 and k_2, did not vary significantly among the three kinds of samples (Table 1).

Thus, while the equilibrium between allosteric states is modified when the nAChR is solubilized in CHAPS, it shifts back to figures typical of the membrane-bound state following addition of A8-35 and dilution of the detergent below its CMC.

4. Discussion

Allosteric properties of two soluble forms of nAChR were examined and compared to those exhibited by the protein in its native membrane environment. CHAPS-solubilized nAChR, with native lipids present but no exogenous lipids added, undergoes allosteric transitions between the basal (B) and desensitized (D) conformational states upon addition of DnsCho. However, as previously observed in other lipid/detergent mixed environments [19,20,35,40], the conformational equilibrium in the absence of agonist is shifted towards the D state. AP-trapped nAChR was prepared by adding amphipol A8-35 to CHAPS-solubilized nAChR and diluting the preparation below the CMC of the detergent. In the absence of AP, this resulted in the precipitation and denaturation of the protein. In its presence, the nAChR remained both soluble and functional. AP-stabilized nAChR retained the lipids associated with the CHAPS-solubilized form and displayed a resting B/D ratio similar to that observed in the membrane environment. This return to native-like allosteric transitions was achieved by mere dilution, without effective removal of the detergent.

These data offer an opportunity to distinguish between a physical and a molecular mechanism for the displacement of nAChR allosteric equilibria. Upon solubilization with CHAPS, the membrane environment of the receptor is replaced with a mixed belt of detergent and endogenous lipids ([26], and present work). Perturbation of the resting equilib-

rium a priori could result from (i) the modification of bulk physical properties (electrostatic field, lateral pressure, viscosity, local dielectric constant, accessibility to water etc.; see e.g. [21,22]), or (ii) the displacement by CHAPS of some of the lipids that interact with the protein in its native environment. After addition of AP and dilution below the CMC of the detergent, the nAChR is surrounded with a mixed layer of AP and lipids, likely interspersed with residual CHAPS molecules. This third medium again is very different from the first two. For instance, the coverage of the protein transmembrane surface by AP is likely to be less dense than that by detergent molecules [4], probably entailing a different accessibility to water. The electrostatic field also is profoundly different. The forces that the nAChR experiences after complexation by A8-35 differ even more profoundly from those imposed upon it by the native membrane environment. In particular, variations of dielectric constant, electrostatic potential or lateral pressure along the transmembrane surface, if present at all, cannot be expected to mimic the strong transmembrane gradients which surround the protein in situ. Its return to native-like allosteric properties therefore is much more likely to result from molecular interactions than from general physical effects. This tallies with the view that the membrane-bound receptor is more sensitive to the chemical composition of the lipid phase than it is to its physical properties [16,23].

Competition with lipids has been proposed as the cause of the local anesthetic-like effects of detergents [35] and fatty acids [16] on membrane-bound nAChR. Similarly, inhibition by the steroid promegestone has been tentatively linked to the displacement of cholesterol from the nAChR/lipid interface [42]. In the likely event that the desensitizing effect of CHAPS involves its displacing lipids from the nAChR surface, two kinds of mechanisms may be considered: (i) binding of lipid molecules promotes the basal state, or (ii) binding of CHAPS favors the desensitized state. This leads to two possible types of explanation for the relieving effect of replacing CHAPS with AP. Both of them rest on CHAPS being displaced from sites it binds to in detergent solution; however, in the first case, it is postulated that the critical factor is that lipids rebind to these sites; in the second, that the detergent be chased from them, whether it be by lipids or by AP.

In conclusion, the present work shows that a protein with such complex conformational transitions as the nAChR recovers, once trapped by AP, functional properties that are much closer to those it exhibits in its native membrane environment than to those observed in detergent solution. Molecular interactions therefore appear to have more importance than bulk physical forces in regulating the functional properties of the nAChR. It will be of interest to further examine the effects of AP on the activity and dynamics of other proteins whose function requires transmembrane conformational changes, and the role of bound lipids and residual detergent in facilitating these movements [5]. As regards the nAChR, AP make it possible to handle it in aqueous solutions in the absence of detergent micelles, under a functional form, while retaining the methodological advantages of an isotropic aqueous medium. This should be of interest for structural, biophysical and pharmacological investigations.

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