RECONSTITUTION OF A FUNCTIONAL ACETYLCHOLINE REGULATOR UNDER DEFINED CONDITIONS

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1. Introduction

The cholinergic receptor protein (AChR) from fish electric organ is a rather well-known protein (reviewed [1]). Sequencing of its main subunit has started [2], its integration to the membrane phase is currently studied by various physical methods, and several of its conformational transitions have been identified and correlated with characteristic changes of membrane permeability (see [1]). Still, an essential question remains:

Which one of the recently identified components of the subsynaptic membrane, polypeptide chains and/or lipids, is necessary and sufficient for the regulation of cationic permeability by acetylcholine (ACh)?

Since a genetic approach to this question is not as accessible with electric fish as it is with *Drosophila* [3], the method we have adopted [4] is to reconstitute a functional ACh-regulator (or receptor–ionophore complex [1]) starting from chemically identified components in solution (reviewed [5]). Preliminary results indicated that the experiment was feasible starting from a complete detergent extract of purified AChR-rich membranes [6,7] or even after purification of the AChR-protein [8,9].

Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; Dns-C_6-Cho, {1-(5-dimethylaminonaphtalene) sulfonamido}*n*-hexanoic acid β-(*N*-trimethyl ammonium bromide); SDS, sodium dodecylsulphate; MPTA, 4-(*N*-maleimido) phenyltrimethylammonium; HTX, histionicotoxin

However, measurement of agonist-sensitive ion fluxes did not appear sufficient to establish the conditions of a complete recovery of the native properties of the ACh-regulator. We therefore attempted to 'reconstitute', first, the binding properties of the membrane-bound AChR [10–12] then, to re-investigate the regulation of ion fluxes under conditions where the native binding properties were preserved.

The interconversion between high and low affinity states of the AChR protein and the effect of allosteric ligands like local anesthetics (see [1]) on this transition were recovered under equilibrium conditions [10,11] and after rapid mixing [12] starting from a detergent extract of purified AChR-rich membranes. These membranes contained several polypeptides:

(1) The well identified 40 000 mol. wt (40 k) chain which carries the AChR-site [13];
(2) A few minor polypeptides of app. mol. wt 50 k and 65 k;
(3) A polypeptide chain of app. mol. wt 43 k [14, 15].

The 43 k protein can be removed from the AChR-rich membranes by alkaline extraction without interfering with the allosteric properties of the AChR protein and in particular its sensitivity to local anesthetics [16].

We present here a method which finally leads to the reconstitution of carbamylcholine sensitive ion fluxes. Under these conditions the allosteric properties of the AChR-protein tested by rapid mixing experiments and the binding of a radioactive anesthetic are preserved in the absence of the 43 k protein.

2. Methods

2.1. *The reconstitution cycle*

The AChR rich membranes were purified from fresh electric organ from *Torpedo marmorata* as in [17] and, in general, stored in liquid nitrogen. Before use, the membranes were thawed, diluted to ~5 mg protein/ml and supplemented with trace amounts of ^{125}I-labelled α-bungarotoxin; then, 3 ml of suspension were centrifuged at 22 000 rev./min for 13 h in a Beckman SW27 rotor on a 37 ml 35–41% (w/w) continuous sucrose gradient and the fractions at 37–39% (w/w) sucrose pooled as 'purified membranes'.

The purified membranes were depleted from the 43 k protein following the method in [16] with the following modifications. The suspension was diluted to 20% (w/w) sucrose, 0.5–1 mg protein/ml and adjusted to pH 11.0 with 1 N NaOH. After 45 min, the membrane suspension was layered on a 25% (w/w) sucrose cushion adjusted at pH 11 and centrifuged at 100 000 × *g* for 2 h. After centrifugation, the 43 k protein remained in solution and the 43 k depleted membranes formed a pellet. The pellet was resuspended in a small volume of buffer I (100 mM NaCl, 10 mM Na-phosphate buffer (pH 7.4), 0.02% NaN_3) and adjusted to pH 7.5 with 1 M Na-phosphate buffer (pH 6.7) (∿ 25 mM final conc.) yielding the 'alkaline-treated membranes'.

The suspension of alkaline-treated membranes was supplemented first with 2-mercaptoethanol to 6‰ final conc., then with 40% (w/w) Na-cholate to yield 3% final conc. (w/w). After shaking for 45 s at room temperature, the mixture was diluted 3 times with buffer I and centrifuged for 20 min at 100 000 × *g* in a rotor 65 of a Beckman ultracentrifuge to pellet the insoluble material. The supernatant was referred to as the 'soluble extract'.

The detergent was separated from the solution of protein by filtration on a Sephadex G-50 column prepared in a 10 ml plastic syringe and equilibrated with *Torpedo* Ringer's solution. The fractions of the excluded volume contained all the AChR and were pooled as the 'reconstituted AChR'.

Alternatively, a dialysis method similar to that in [7] was used. A mixture of 10% asolectin and 10% cholate, in buffer I was added to 100–500 μl of membrane fragments (~10 μM α-toxin binding sites) to obtain a final 3% cholate concentration; 3–6‰ 2-mercaptoethanol could be added at this stage. After stirring for 30–60 s at room temperature, the mixture was diluted to 1% cholate with buffer I and centrifuged as above. The supernatant was dialysed ~24 h against 500–1000 vol. of deaerated buffer I in Spectra/Por 2 dialysis tubing (mol. wt cut off 10 000) under a nitrogen atmosphere.

2.2. *Stopped flow experiment with Dns-C_6-Cho*

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. 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 multiexponentials, and both amplitudes and rate constants determined.

2.3. *Equilibrium binding of [^{3}H]trimethisoquin*

Binding of [^{3}H]trimethisoquin was measured by a centrifugation method using a Beckman Airfuge and polyethylene tubes. The AChR containing sample was diluted in *Torpedo* physiological solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na-phosphate (pH 7.0)) to 3–4 μM in α-bungarotoxin binding sites, 70 μl incubated with [^{3}H]trimethisoquin (0.02 Ci/mmol) for 60 min and centrifuged at 130 000 × *g* for 15 min; 10 μl duplicate aliquots taken before and after centrifugation were counted in 4 ml Bray's scintillation medium.

2.4. *Ion fluxes*

Agonist-dependent influx of $^{22}Na^+$ was measured on these preparations by an adaptation of the efflux method in [18]. In a typical experiment 5 μl carrier-free ^{22}NaCl in water were added at 0°C at time zero to 100 μl of reconstituted material; 10 μl aliquots

were taken at increasing times, diluted into 5 ml ice-cold buffer and immediately filtered upon HAWP Millipore filters; after washing with 2 × 10 ml ice-cold buffer, the filters were placed into vials and counted for γ emission in a CG 30 Intertechnique counter. Agonists were added as concentrated solutions, the resulting dilution factor being usually 1 or 2%; the same volume of buffer was added in control experiments.

2.5. *Polyacrylamide gel electrophoresis*

SDS–polyacrylamide gel electrophoresis was done with 1.1 mm thick, 10% acrylamide slab gels by the method in [17]. Destained gels were scanned with a Vernon gel scanner.

2.6. *Chemicals*

Dns-C_6Cho 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 [19]. ^{125}I-labelled α-bungarotoxin was from NEN; prilocaine hydrochloride and trimethisoquin (1-β-trimethylaminoethoxy) 3-*n*-butylisoquinoline hydrochloride) were a gift from Lab. Roger Bellon (France); Na cholate (analytical grade) was from Merck. ^{22}NaCl was from the Radiochemical Centre, Amersham. [^{3}H]Trimethisoquin was synthesized by G. Waksman and B. Roques and tritiated in Dr B. Pichat's lab. at CEA Saclay. Histrionicotoxin was a gift of Dr G. Kato. Asolectin was from Associated Concentrates (USA).

3. Results and discussion

3.1. *The reconstitution cycle*

Three critical properties of the ACh-regulator have been monitored during the reconstitution cycle:

(1) The rapid binding kinetics of a fluorescent agonist, Dns-C_6-Cho, recorded in a Gibson-Durrum stopped-flow apparatus equipped for fluorescence detection;
(2) The binding of a radioactive local anesthetic [^{3}H]methyl-trimethisoquin;
(3) The regulation of ion fluxes through lipid vesicles containing the AChR.

Four preparations of the AChR were used (see section 2.1.):

(1) The purified membranes, with a specific activity of 3–4 μmol of ^{125}I-labelled α-bungarotoxin sites/g protein (table 1), yielded, after electrophoresis in one dimension on SDS–polyacrylamide gels, two dominant polypeptide bands present in approximately equal amounts (see [17]): the 40 k chain which can be covalently labelled by [^{3}H]MPTA Karlin's affinity reagent of the AChR site [13] and the 43 k chain. The 50 k and 66 k bands were systematically observed in most of the preparations, but in variable proportions and in small quantities.
(2) Treatment of the purified membranes at pH 11 yielded the alkaline-treated membranes, which, in agreement with the findings of Neubig et al. [16], were depleted of the 43 k protein but still contained

Table 1
AChR-sites and local anesthetic binding sites in the reconstitution cycle

	^{125}I-labelled α-bungarotoxin binding sites (μmol/g protein)	[^{3}H]Trimethisoquin binding sites (μmol/g protein)	[^{3}H]Trimethisoquin sites over ^{125}I-labelled α-bungarotoxin sites
Purified membranes	3.8	2.6	0.68
Alkaline-treated membranes	6.9	5.3	0.77
Reconstituted AChR	8.0	6.2	0.77

Saturable [^{3}H]trimethisoquin binding sites were determined (see section 2) in the presence of carbamylcholine (5×10^{-5} M). The amounts of [^{3}H]trimethisoquin bound in the presence and in the absence of HTX (10^{-4} M) were plotted as a function of free [^{3}H]trimethisoquin concentration: the difference between the two curves reaches a plateau which gives the concentration of local anesthetic binding sites. ^{125}I-labelled α-bungarotoxin binding sites were measured with a Sephadex G-75 column as in [17]

the 40 k, 50 k and 66 k bands. The specific activity of these membranes in ^{125}I-labelled α-bungarotoxin sites ranged between 6–8 μmol/g protein.

(3) The soluble extract was obtained by adding Na-cholate to the alkaline-treated membranes to 3% final conc. (w/w). The mixture was then diluted to 1% and centrifuged at 100 000 × *g* for 20 min to pellet the insoluble material. The polypeptide composition (fig.1) of the supernatant, in general, did not differ from that of the alkaline treated membranes.

(4) The elimination of the detergent from the soluble extract by filtration on a Sephadex G-50 column or by dialysis yielded two different states of the AChR depending whether or not the extract was supplemented with additional lipids (asolectin). In the first instance, the preparation was referred to as 'reconstituted AChR', in the second as 'reconstituted vesicles'. The polypeptide composition and specific activity of these two preparations was identical to that of the soluble extract. The yield in ^{125}I-labelled α-bungarotoxin sites throughout the reconstitution cycle was ~ 60%.

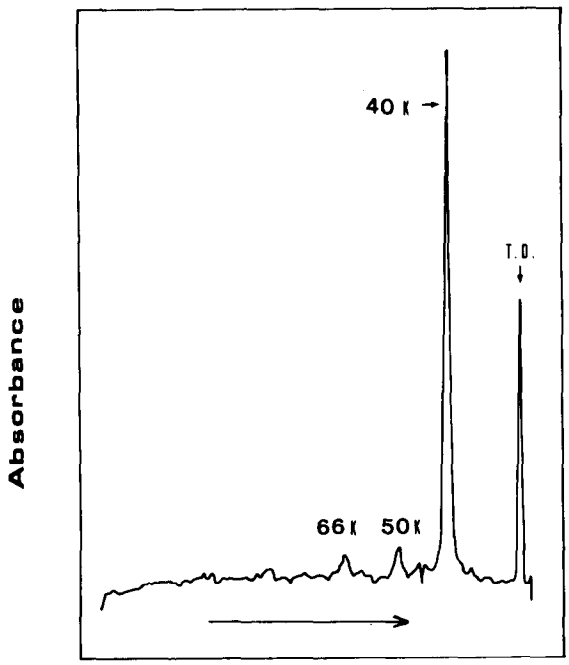

Fig.1. Polypeptide composition of the 'soluble extract' analyzed by SDS–polyacrylamide gel electrophoresis as in section 2. T.D., tracking dye.

3.2. *Rapid kinetics of interaction of Dns-C_6-Cho with the AChR-site in the reconstitution cycle*

In fig.2 are presented stopped-flow traces obtained by rapid mixing of the fluorescent agonist Dns-C_6-Cho [19] with the purified alkaline-treated membranes or with the reconstituted AChR. In all these three instances, the recordings appear rather similar. The three relaxation processes, fast, intermediate and slow, identified with the native membranes [20] were observed, and interpreted in terms of the interactions of Dns-C_6-Cho with high and low affinity binding sites and of an interconversion of the low to the high affinity sites in the presence of this agonist. Accordingly, after reconstitution, a significant fraction of the AChR-sites were in a low affinity state (~70%) and could undergo the slow conformational transition previously related to the 'desensitization' of the membrane response to cholinergic agonists (reviewed [1]).

Local anesthetics and other non-competitive blockers of the physiological response to ACh have been shown to modify the kinetic parameters of Dns-C_6-Cho interaction with purified AChR-rich membranes in the following manner: they increase the amplitude of the rapid relaxation process and the rate constants of the intermediate and slow processes [20]. Figure 2 shows that the same effects are observed in the case of trimethisoquin with alkaline-treated membranes and reconstituted AChR. Conclusively, and in agreement with the findings of Neubig et al. [16], the 43 k protein which is missing in these two preparations, does not take part in the allosteric interaction between local anesthetic binding site and AChR-site.

3.3. *Equilibrium binding of [^{3}H]trimethisoquin in the reconstitution cycle*

The binding site for local anesthetics and non competitive blockers has been labelled by radioactive derivatives of potent local anesthetics such as [^{3}H]trimethisoquin [21,22]. Binding curves of [^{3}H]trimethisoquin have been established with the three particulate preparations described in the recon-

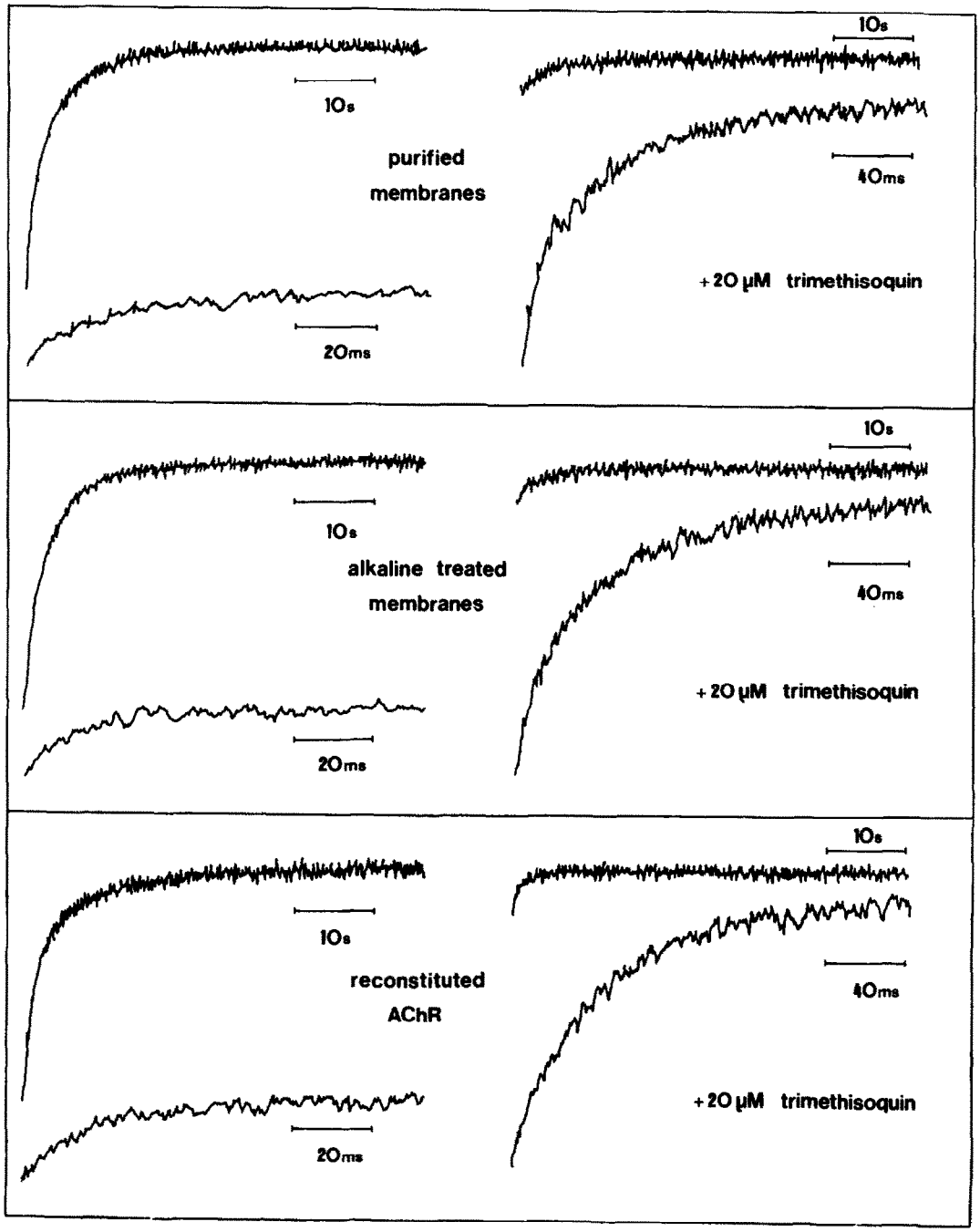

Fig.2. Single-shot traces of stopped-flow experiments: change of fluorescence intensity after rapid mixing of a solution of Dns-C_6-Cho with purified membranes (top), alkaline-treated membranes (middle) and reconstituted AChR (bottom); effect of trimethisoquin. λ_{ex} = 290 nm, λ_{em} > 540 nm (cf. section 2); 1:1 mixing of a solution of Dns-C_6-Cho in *Torpedo* saline solution (3 μM) with purified membranes, alkaline-treated membranes and reconstituted AChR (∿ 0.4 μM α-toxin binding sites) in *Torpedo* saline solution in the absence (left) or presence (right) of 20 μM trimethisoquin; rapid relaxation processes are represented on expanded time scales.

stitution cycle in the presence and in the absence of histrionicotoxin (HTX). Table 1 shows that with the native membranes and in presence of the cholinergic agonist carbamylcholine, the number of high affinity binding sites from which [^{3}H]trimethisoquin is displaced by HTX (see [21,22]) was ~0.7-times the number of ^{125}I-labelled α-bungarotoxin sites. Interestingly, the same values were found with the alkaline-treated membranes and the reconstituted AChR. From a series of experiments carried out with detergent solubilized 43 k protein and quinacrine it was inferred that the 43 k protein bound local anesthetics [14]. The present data confirm that these sites are not the regulatory sites involved in the allosteric interaction between local anesthetics and cholinergic agonists.

3.4. *Recovery of agonist-sensitive ion fluxes*

When the last step of the reconstitution cycle was carried out in the presence of a complex lipid mixture, such as asolectin, closed vesicles were obtained. The rapid kinetics of interaction of Dns-C_6-Cho with the AChR protein integrated to these reconstituted vesicles did not significantly differ from those obtained when the reconstitution step was performed in the absence of exogenous lipids. As shown in fig.3, these vesicles exhibit a rather low permeability to $^{22}Na^+$, but carbamylcholine markedly enhanced the influx of $^{22}Na^+$ and this response was abolished by preincubation with an excess of α-bungarotoxin. The effect of carbamylcholine on $^{22}Na^+$ influx was observed with the 7 preparations tested and therefore, appears reproducible.

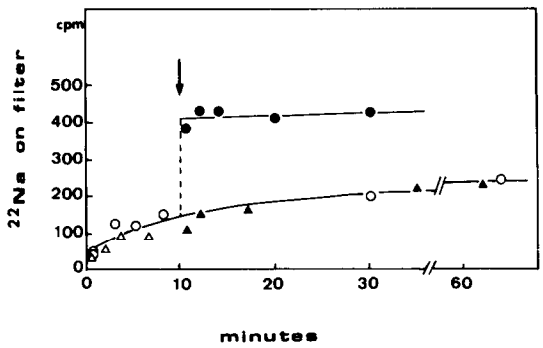

Fig.3. Concentrated ^{22}NaCl was added at t=0 to a suspension of reconstituted vesicles preincubated (△) or not (○) with a ~20-fold excess of α-bungarotoxin; 20 μl aliquots were diluted into ice-cold buffer at the times indicated and rapidly filtered. At t=10 min (arrow), the remnant of the suspension was supplemented (●,▲) or not (○) with enough concentrated carbamylcholine to give 4 × 10^{-4} M final conc.

Since the 40 k chain is largely the dominant polypeptide in the reconstituted material, the most simple interpretation of the present data is that the 40 k chain carries both the ACh-receptor site and the ACh-ionophore. However, small amounts (< 10%) of the 50 k and 66 k chains were still present in the reconstituted vesicles; a small number of functional receptor–ionophore complexes per vesicle might be sufficient to give a measurable influx of $^{22}Na^+$ and the 'contaminating' peptides might precisely be part of these complexes. This possibility has to be considered and experimentally tested. Future work should also include investigations on an eventual role of the endogenous lipids which remain associated with the AChR protein during the reconstitution cycle.

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