INTRINSIC FLUORESCENCE OF THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR : ITS QUENCHING BY SUBERYLDICHOLINE

F. J. Barrantes

Max-Planck Institute für Biophysikalische Chemie, 3400 Göttingen, W. Germany

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Abstract: The intrinsic fluorescence of the acetylcholine receptor from *T. marmorata* decreased upon exposure to the agonist suberyldicholine. The kinetics of this saturable process have been followed with rapid fluorimetric techniques in conjuction with α-toxin binding inhibition studies. The existence of discrete forms of the receptor, characterised by different fluorescence states and affinities towards the agonist account for the experimental observations. The most conspicuous change in the protein fluorescence of the membrane-bound receptor occurred within the same time range as the ligand-induced increase in affinity. These parallel changes are consistent with the phenomenon of pharmacological "desensitisation" observed *in-vivo* in the cholinergic synapse.

The nicotinic acetylcholine receptor (AChR) is an integral membrane protein[1], and although valuable information has been gained from working *in-vitro* with the isolated macromolecule[2], the study of the AChR in its natural membrane environment constitutes a closer approximation to the understanding of receptor-mediated physiological phenomena. Our knowledge of AChR-ligand interactions at this level of organisation has been mainly restricted to equilibrium binding parameters of various cholinergic effectors, and to a lesser extent, to the kinetics of the quasi-irreversible α-toxins from snake venoms[2].

The application of fluorescence techniques to the above problem has so far been undertaken with the aid of extrinsic fluorescent probes

bearing different degrees of specificity towards the cholinergic system[3-5]. Recent attempts have been made to use the intrinsic fluorescence of the solubilised AChR; in spite of its relatively high quantum yield, no changes were detected upon mixing with various cholinergic ligands[6].

In the present study fluorescence kinetic techniques show that the potent agonist suberyldicholine (SubCh) quenches the *intrinsic* fluorescence of the membrane-bound AChR from *T. marmorata*. Parallel α-toxin binding inhibition studies confirm that these fluorescence changes are accompanied by an increase in the affinity of the receptor towards SubCh. At equilibrium, the apparent dissociation constant for this ligand is practically equal to that of acetylcholine[2]. The kinetic and equilibrium parameters distinguish between two extreme states of the AChR: a resting, native state and a "desensitised" form, in accordance with electrophysiological experiments in the motor endplate[7] and with more recent studies on [^{22}Na]- efflux[8] or fluorescence measurements [9] in *Torpedo* microsacs.

Material and Methods.

AChR-rich microsacs from the electric organ of *T.marmorata* were prepared essentially as described by Cohen et *al*[10].Their toxin-binding capacity was determined by the Millipore method[11], and the inhibition of the initial rate of α-toxin binding according to Weber and Changeux[12]. Fluorescence measurements were performed in the apparatus designed in this laboratory[13], with the additions given below. The exciting light from a 200 W He/Xe arc was passed through a Zeiss monochromator set at 296 nm, a 295.1 nm interference filter (Corion Corp.) and a light chopper operating at 50 Hz. The emitted fluorescence was measured either as the entire spectrum above 320 nm or as the fluorescence detected with quartz fibre optics at 320 nm with a Heath

EU-700 monochromator (2 nm band pass). The ratio of the fluorescence signal to the exciting light was averaged over 0.4 to 1 sec with a phase-sensitive lock-in amplifier, temporarily stored in a Fabritek 1074 signal averager, and finally transferred to a PDP 11/20 minicomputer for analysis. Stopped-flow experiments were carried out in the same apparatus[13] directly on-line with the minicomputer for real-time data acquisition[14]. Analysis of the data was performed with a non-linear regression programme written by Dr. R. Clegg. Further experimental details will be published elsewhere[15].

Results.

AChR-rich microsacs from *T. marmorata* have a fluorescence emission spectrum characteristic of proteins containing tryptophan in a hydrophobic environment, as evidenced by the maximum around 330 nm. The effect of SubCh on the intrinsic fluorescence could be uninterruptedly followed, as shown in Fig.1, from 2-5 sec up to several minutes. This in turn enabled i) the accurate determination of the initial and final ligand-induced fluorescence values under identical instrumental conditions; ii) the successive addition of ligand (Fig.1,*c,d*) and iii) the evaluation of the fluorescence changes due to dilution and spontaneous decrease of the protein fluorescence. The latter effect,probably due to photo lysis, was quantitated in control experiments and substracted from each record.

Addition of SubCh affected the intrinsic fluorescence in a characteristic manner: the change consisted of a time-dependent, saturable decrease of the fluorescence emission (Fig.1). The specificity of the phenomenon is based upon the following facts: a) preincubation of the membranes with saturating amounts of native α-toxin prevented the ligand-induced response (Fig.1 *a*);b) denat-

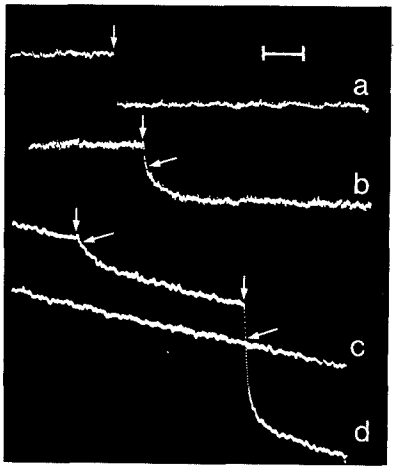

Fig.1 Effect of SubCh on the intrinsic protein fluorescence of AChR-rich microsacs from *T. marmorata*. Microsacs were diluted (3 : 100) in the physiological solution in 0.7 mm quartz cuvettes (1.7 ml) to a final protein concentration of 20 to 50 µg/ml. A special "injection-stirring" device was then inserted into the cuvette. The device consists of a rotary glass stirrer driven by a motor at a controlled speed, a narrow-bore stainless steel tube connected to an external 10 µl precision syringe, propelling the ligand solution into the mixing area, and an air exhaust tube. The tubing and stirrer, bored through a Teflon stopper, were positioned outside the illuminated area. Injection and mixing of the small ligand samples, under continuous illumination and monitoring of the fluorescence signal, could be achieved in 2 - 4 sec with this assembly. In control experiments the toxin binding activity was measured both before and after submitting the membranes to UV-irradiation and stirring for similar periods of time. *a*) Injection of 100 nM SubCh to a microsac suspension (50 nM in [^{3}H]-α-toxin sites) preincubated with a 20-fold molar excess native α-toxin. Only the dilution is observed. Each point corresponds to the fluorescence emission integrated over 400 millisec, and each trace consists of 1024 such points. The horizontal bar corresponds to 52 sec in this trace. *b*) Oscillographic display showing the decrease in intrinsic fluorescence upon addition of 88nM SubCh to 50 nM AChR-rich microsacs. An exponential decay with a half-time of 59 $\pm$ 8 sec and an amplitude of -0.45% is observed. The

uration by heating also abolished the ligand effect; c) the amplitude of the SubCh-induced quenching was proportional to the specific activity (α-toxin sites), but not to the total protein content or acetylcholinesterase activity of the various preparations tested. Similar ligand-induced changes have been observed with acetylcholine and carbamylcholine in the same preparation[15].

In order to study the SubCh-induced responses under conditions of pseudo-first order kinetics, i.e. [SubCh] $\gg$ [AChR], the stopped-flow fluorimetric technique was employed. As shown in Fig. 2, the phenomenon could be explored over a wider range of ligand concentration and the responses could be followed accurately after the initial mixing (dead-time of the instrument was 5 millisec) up to 3-4 min.

The rate of the SubCh-induced changes increased as a function of ligand concentration and temperature; they reached half-saturation at about 6 μM (4°C) and plateau in the region of 12-60 μM ligand concentration. Higher concentrations of acetylcholine or carbamylcholine were needed to elicit similar responses[15], in accordance with the relative pharmacological potencies of the three agonists[16].

An indirect, though very sensitive technique, the measurement of the initial rate of α-toxin binding provided additional insight into the SubCh-AChR interactions. As shown in Fig. 3, the concentration of ligand necessary for 50% inhibition of the apparent rate of toxin binding, determined at zero time (simultaneous addition of

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vertical arrow indicates the time of injection; the oblique arrow, the amplitude decrease accounted for by dilution. The horizontal bar corresponds in this case to 52 sec. *c)* Two successive injections of SubCh, the first one, with a response characterised by a τ = 210 ± 10 sec and an amplitude of -0.28% was produced by 30 nM ligand on 50 nM receptor sites; the second one corresponds to 0.6 μM SubCh and 50 nM AChR and has a decay time of 40 ± 8 sec and an amplitude of -1.25%. The bar indicates 135 sec for this trace. *d)* Continuation of trace *c* .

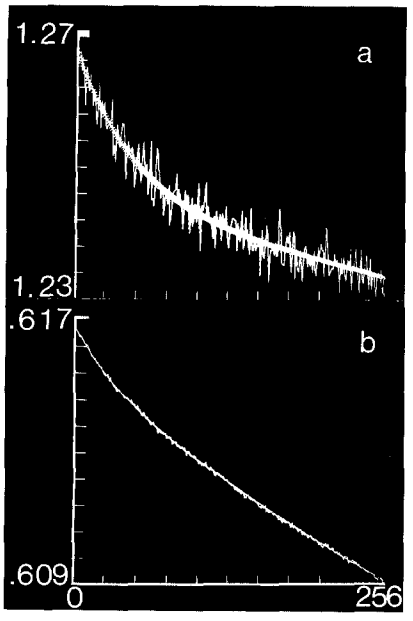

Fig.2 Individual stopped-flow records of the fluorescence decay induced by suberyldicholine on AChR-rich microsacs. The ordinate corresponds in both graphs to relative fluorescence intensity; the time axis, showing the 256 data points collected in each case, correspond in *a)* to 22.8 sec and to 178.24 sec in *b)*. Temperature: $4^{o}C$.
a) 6.05 μM SubCh, 20 nM receptor sites, 22 μg/ml of membrane protein. The solid line through the experimental points corresponds to the exponential decay calculated for a $\tau = 3.7 \pm 0.5$ sec and an amplitude of $-1.7 \pm 0.3\%$, superimposed on a linear baseline drift. *b)* 1.21 μM SubCh, 22 nM receptor sites, 21 μg protein/ml. The decay time is in this case 23.8 ± 0.8 sec, the amplitude -0.9 ± 0.1 %. The base-line linear drift is accounted for in the analysis. Wavelength of excitation, 295.1 nm; protein fluorescence signal was collected above 320 nm (Schott WG320 filter, Mainz). The fluorescence signal was directly transferred via a coaxial line and an analog-digital converter to a PDP 11/20 minicomputer operating on-line for real-time data acquisition [14]. Six successive sets of data points, each consisting of 256 averaged fluorescence readings were uninterruptedly collected from 5 millisec (dead-time of the instrument) up to 3-4 min. Analysis of the data was performed using a non-linear regression programme written by R.Clegg.

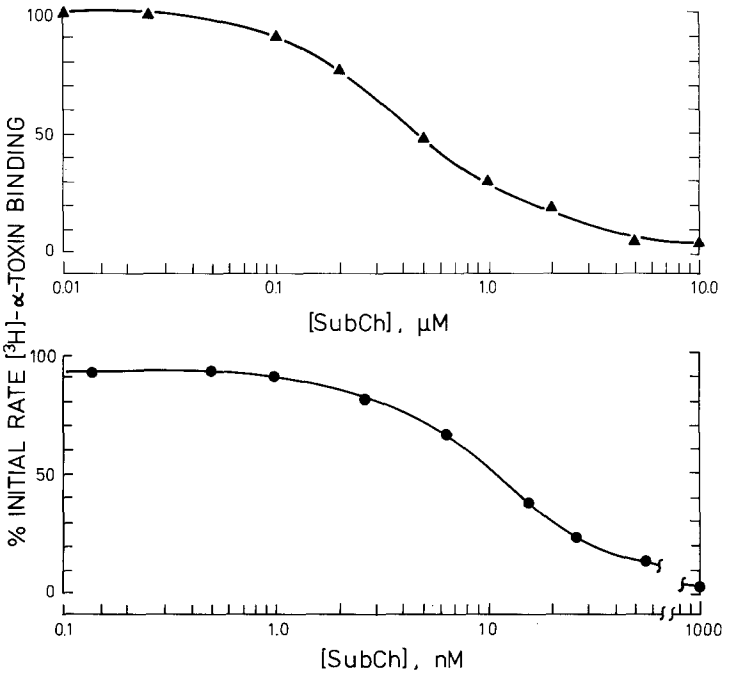

Fig. 3 Effect of SubCh on the apparent initial rate of $|^{3}H|$-α-cobrotoxin (2.92 Ci/mmole) binding to AChR-rich microsacs. The upper graph shows the inhibition produced by SubCh added simultaneously with the toxin to the microsac suspension (i.e. no preincubation). Each point corresponds to the apparent initial velocity of the toxin-receptor association reaction determined in duplicate experiments according to ref. 12. The reaction was followed over the initial 5 min. The lower graph corresponds to the inhibition exerted by SubCh after equilibration for 30 min with the AChR-rich membranes. It was determined that 30 min sufficed to elicit the maximal effect for the whole ligand concentration range explored (0.13 nM- 10 μM). Notice the difference in the units of ligand concentration in the two curves. The preincubation with SubCh (lower graph) led to an apparent increase in the affinity of the receptor towards SubCh of about 50-fold. In both series of experiments AChR concentration was 5.0 nM and $|^{3}H|$-α-toxin 1.6 nM. Temperature in both cases was 20°C.

SubCh and α-toxin to the microsacs) was 0.5 ± 0.03 μM *(upper graph)* i.e. significantly higher than that determined after long preincubation of the microsacs with SubCh (30 min) prior to toxin addition (10 ± 2 nM, *lower graph)*. This substantial change in the affinity of the AChR for agonists can be interpreted as reflecting a change in the state of the receptor elicited by the ligand itself [6,17]. In the case of SubCh, the kinetics of onset of the affinity change could be followed and compared with the observations of the fluorescence experiments. For instance, with 10 nM SubCh the half-time for achievement of maximal inhibition of the apparent toxin binding rate was 120 sec, whereas with 50 nM ligand the same effect was reached in about 55 sec, in both cases following an exponential behaviour. This is, in a first approximation, in agreement with the rates of the SubCh-induced fluorescence changes.

Discussion.

In the present report the kinetics of SubCh-induced changes have been followed using both the intrinsic fluorescence of the AChR and the inhibition of α-toxin binding. A similar time-course was observed in the intrinsic fluorescence quenching and the apparent increase of the affinity of the AChR (∿50-fold), suggesting a common mechanism elicited by the agonist. The simplest explanation for these observations and the similar changes produced by acetylcholine and carbamylcholine[15] is that the AChR-agonist interactions consist of a rapid initial step followed by a slower isomerisation of the form:

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_4}{\overset{k_3}{\rightleftharpoons}} DL \underset{k_5}{\overset{k_6}{\rightleftharpoons}} D + L$$

where R is the resting, unliganded AChR, RL the initial complex, and D and DL the "desensitised" forms (see below). The α-toxin inhibition studies provided a lower estimate of the *apparent* dissociation constant for the initial step, i.e. $K_1 \sim 0.5$ μM (Fig. 3).

In the fluorescence experiments the existence of this fast step is manifested indirectly in the decline of the kinetic amplitudes associated with the slow decay times at high enough ligand concentration, as is also the case with acetyl- or carbamylcholine[15]. After the initial step(s) the equilibrium is shifted towards the DL form, as evidenced by the 50-fold affinity enhancement occurring upon exposure to the agonist. At equilibrium, the apparent dissociation constant for SubCh is 10 nM, i.e. as low as that of acetylcholine[2]. Since in untreated membranes the R form predominates[2], and the unliganded form D is negligible in all cases, it is not surprising that the slow isomerisation RL → DL was the most conspicuous reaction step in the agonist-AChR interactions, tentatively assigned to the phenomenon of "desensitisation". This phenomenon, the decline of the agonist-induced response despite application of the ligand[7,17,2] has been observed *in-vivo* in *Torpedo* electroplax[18] and also *in-vitro* in the microsac preparation from the same species[8,9]. It was originally interpreted as a structural transition of the AChR[7] but the evidence for such a structural change remained hypothetical[7-9]. It is possible that the SubCh-induced fluorescence changes, as well as those induced with other agonists[15] bear a common structural basis with the induction of the high-affinity form of the AChR, this in turn constituting the molecular mechanism of desensitisation. Studies now in progress are aimed at characterising this phenomenon in kinetic and structural terms.

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