Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 766-774

FAST KINETICS OF ANTAGONIST-ACETYLCHOLINE RECEPTOR INTERACTIONS: A TEMPERATURE-JUMP RELAXATION STUDY

Y. Tan and F.J. Barrantes
Max-Planck-Institut für biophysikalische Chemie
3400 Göttingen-Nikolausberg, West Germany

Received December 10,1979

Summary: The interactions of an antagonist with the membrane-bound acetylcholine receptor from Torpedo have been studied using the temperature-jump relaxation method. The fluorescence emission of the antagonist, a pyrenyl-choline derivative, excited by energy transfer from the protein chromophores, enabled the observation of two relaxation processes in the lower millisecond time range. The concentration dependence of the relaxation times and associated amplitudes could be accounted for by a reaction scheme involving the binding of two antagonist molecules and the subsequent isomerization of the complexes. Both binding steps appear to be diffusion-controlled and the second isomerization rate limiting. Competitive and non-competitive antagonism are discussed in relation to the proposed reaction mechanism.

Recent advances in the study of the reaction mechanisms between the acetylcholine receptor (AChR)[1] and cholinergic ligands in vitro have been accomplished by applying kinetic techniques (see review in (1)). In most cases extrinsic fluorescent probes have been employed in conjunction with the stopped-flow technique (2-7). Absorption spectroscopy has been used in one case (8). A slightly more direct approach utilized the intrinsic fluorescence of the membrane-bound AChR and cholinergic agonists of well characterized activity, including the natural neurotransmitter acetylcholine (9-11). From other potentially useful approaches or probes (see e.g. ref. (12)) only fragmentary and qualitative information is available. Since the introduction of dansyl-choline (13), and the subsequent demonstration of its mixed agonist-local anaesthetic action in vivo (14), it became apparent that the pharmacological characterization of extrinsic fluorescent probes is an absolute prerequisite to the performance of any spectroscopic study in vitro. This prerequisite has been met for example in a later study (15) in which pyrene butyrylcholine was synthesized, having in mind the high molecular weig

[1]Abbreviations used are: AChR, acetylcholine receptor; compound III, 2-[4-(1-pyrenyl)-butyrylamino-ethyl]-trimethylammonium perchlorate.

0006-291X/80/030766-09$01.00/0

of the AChR oligomer and the need to match its rotational relaxation time with a chromophore of particularly long lifetime such as pyrene. This (15) and other (16) compounds of the series were found to be competitive antagonists of the nicotinic AChR.

In the present work one of these compounds is used to study the kinetics of the interaction with the membrane-bound AChR from Torpedo marmorata by means of the temperature-jump relaxation technique. The simplest reaction mechanism accounting for the observed kinetics is discussed in relation to the available electrophysiological information on the action of this type of cholinergic blocking agent.

Material and Methods.

Preparation of membrane fragments, ^{3}H-α-toxin binding assay and toxin rate measurements in the presence and absence of compound III, the pyrenyl derivative (16), were carried out as described in ref. 11. Typically, specific activities of 0.7-1.2 nmole ^{3}H-α-toxin sites per mg protein were obtained. The membrane fragments were stored in 1.2 M sucrose containing 0.02% NaN_3 and 0.1 mM phenylmethane sulphonylfluoride at 4°C and used within four days of preparation. Absorption measurements were carried out in a Cary model 118 spectrometer. Fluorescent measurements were made in the fluorimeter described in (11) adapted for temperature-jump measurements using fluorescence detection. The excitation wavelength was 297 nm (Heath monochromator+interference filter, Corion Corp.) and the fluorescence emission was collected above 370 nm with a Schott KV370 filter in front of the photomultiplier. Data collection started 20 μsec after the Joule heating produced by an 18.3 kV discharge; the rise in temperature was 3.2°C. The 7mm pathlength cell, with a heating volume of 700 μl, was thermostated at 20°C with a circulating bath. Fluorescence signals were amplified, digitized and transferred to a PDP 11/20 minicomputer at 40 μsec intervals, and subsequently averaged and analyzed in the same computer using programmes written by L. Avery. The postulated reaction mechanism was fitted with theoretical functions derived from the scheme 1 (see below) using a non-linear least-square programme written by J. Chandler.

RESULTS AND DISCUSSION

The pyrenyl-choline derivative (compound III, chemical formula in Fig. 1) used in this study possesses an amide bond replacing the ester linkage of the previously introduced derivative (15). This change made the probe resistant to esterase hydrolysis, which was observed both in vivo in the neuromuscular junction and in vitro with acylcholine acyl-hydrolase (E.C. 3.1.1.8).

The spectroscopic properties of the new fluorescent probe were found to be similar to those of other pyrene derivatives: The absorption spectrum (Fig. 1) shows

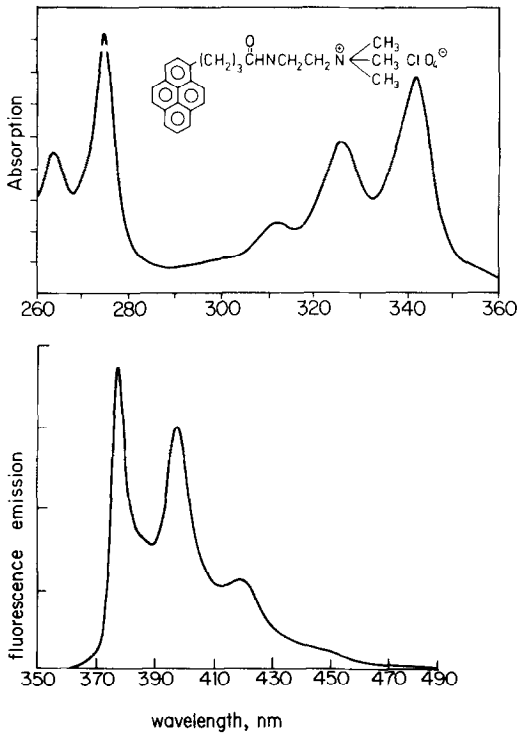


Figure 1. Structural formula, absorption (upper) and fluorescence emission (lower) spectra of compound III ($\varepsilon_{342.5\ nm}$ = 30 123) in Torpedo saline.

the characteristic allowed vibronic bands and the relative minimum around 290 nm, which made possible the use of energy transfer from the intrinsic chromophores of the AChR-rich membranes (absorption maximum around 295 nm, ref. 11) to the probe.

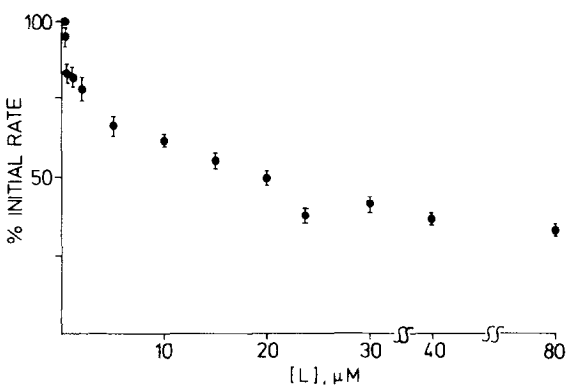


Figure 2. Concentration dependence of the inhibition of ^{3}H-α-cobrotoxin apparent initial rate of association by compound III. Toxin and receptor concentrations were 5 nM and 1.5 nM respectively; temperature 20°C. Each point is the mean of at least two independent experiments. The apparent rate of toxin association was calculated as in ref. 11.

The fluorescence emission spectrum, upon excitation of the probe at 342.5 nm (free probe) displayed the typical transition bands of pyrene moieties in aqueous media, dominated by the 373 nm peak.

Compound III inhibited the initial rate of ^{3}H-α-cobrotoxin-AChR complex formation. The inhibition followed the pattern typically found with antagonists of the cholinergic system, namely the lack of the time dependence characteristic of the agonists (10, 3, 18). In addition, compound III blocked the response to ionophoretically-applied acetylcholine in chick muscle cells in culture and in the adult neuromuscular junction (16). Half-inhibition of toxin apparent rates occurred at about 15-20 μM ligand (Figure 2), whereas the in vivo blockade was apparent at about 2 μM probe (16).

The kinetics of the interaction of compound III with the AChR could be characterized by the use of fast relaxation techniques under energy transfer conditions. Temperature-jump experiments were conducted in a limited range of ligand concentration, attending to i) the results from steady-state fluorescence titrations (16), ii) the thermodynamics governing the observation of relaxation signals using the above perturbation technique, and iii) the minimization of the inner filter effect due the high extintion coefficient of the probe (Fig. 1). From (i) an apparent equilibrium dissociation constant of compound III of about 2 μM was obtained (16).

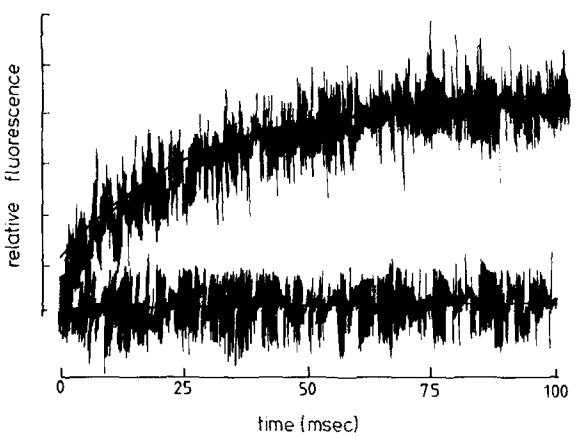


Figure 3. A typical temperature-jump single record of AChR-rich membranes (50 nM in toxin sites, 55 μg protein/ml) in the presence of 2 μM compound III. Top trace: control experiment under the above conditions. The two solid lines running through the data are a single and a double exponential function generated by the fitting routine; notice the poor nature of the single exponential fit though the data is very noisy. The double exponential fit yielded τ_I (fast reaction rate)= 4.1 msec and τ_{II} (slow process)= 42.6 msec. The lower trace was obtained with membranes preincubated with 2 μM bungarotoxin prior to the relaxation experiment in the presence of 2 μM compound III. The curve passing through the data is hand-drawn; notice the absence of relaxation signals.

The fluorescence kinetic signal observed upon abruptly raising the temperature by 3.2°C consisted of at least two relaxation processes. As shown in Fig. 3, even the visual criteria suffice to reject a single-exponential fit to the experimental data; multiexponential analysis confirmed the need to use at least two exponential functions to describe the observed kinetics. As can be seen in Fig. 3 (bottom trace) both relaxation signals completely disappeared upon preincubation of the membrane fragments with 2 μM bungarotoxin. Further control experiments, such as suddenly changing the temperature of the probe solution or the membrane suspension did not produce relaxation signals. The reproducibility of the observed responses was attested by the identical trends in the behaviour

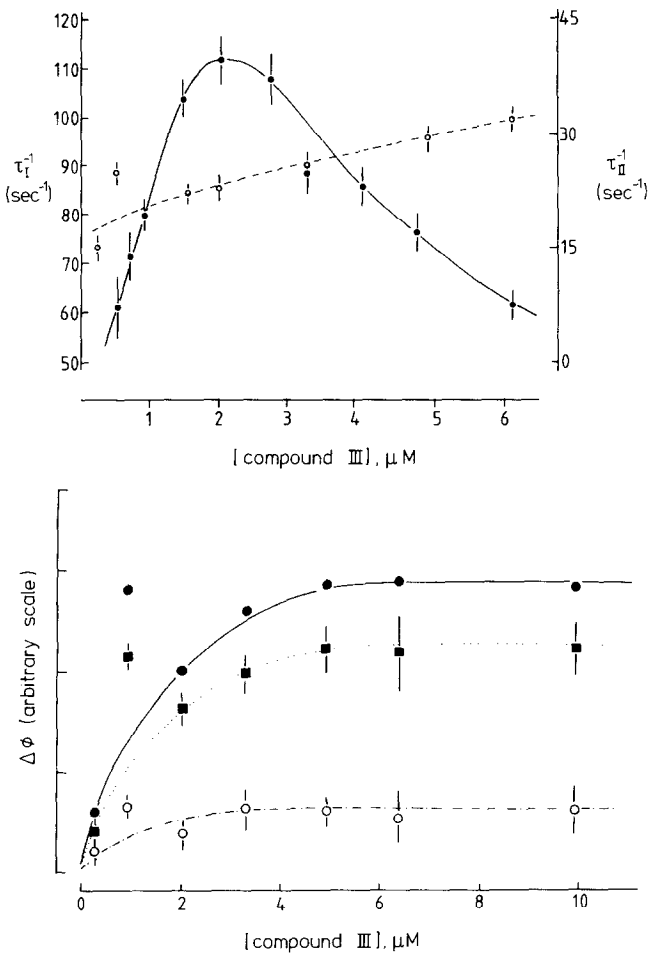
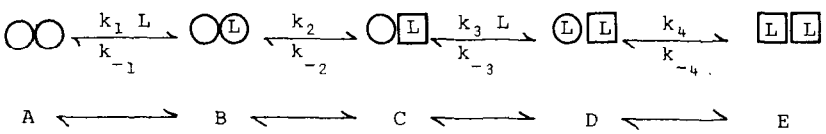


Figure 4. Dependence of the inverse of the relaxation times (upper graph) and of the amplitudes (lower graph) on the concentration of compound III. The filled circles represent the reciprocal rates of the fast process (τ_I) and the empty circles the slow process (τ_{II}). The amplitude associated with τ_I (■) and with τ_{II} (o) are arithmetically added to yield the total amplitude (●). Lines are hand-drawn. Each point is the average of at least nine temperature-jump records.

of the relaxation times and associated amplitudes in four series of experiments performed on membrane fragments of different specific activities.

The concentration dependences of the relaxation times (Fig. 4) indicated that what is being observed cannot be explained by a simple bimolecular ligand-AChR interaction, in which case one would expect linearly increasing reciprocal reaction rates with increasing ligand concentration. The facts that a) the fast reaction rate reached plateau values after passing through a maximum; b) the slow process showed a hyperbolic concentration dependence, and c) both kinetic amplitudes saturated at high ligand concentration, together with d) the fact that the AChR is a multimeric protein (1-7), are all consistent with the hypothesis that the AChR-compound III observed relaxations are associated with isomerizations of the protein. Under the assumption that the binding reactions are faster than the isomerization steps (being at equilibrium in the time scale explored), and taking into account that the concentrations of compound III used were much higher than those of the AChR sites (buffering ligand conditions), the simplest reaction mechanism to explain the observed dependence of reciprocal reaction rates on ligand concentrations turned out to be a sequential scheme of the type:



(1)

where the first isomerization (B ⇌ C) is faster than the second one (D ⇌ E) (the reverse does not hold, see ref. 20). According to this scheme the following dependence of the relaxation time should be observed:

$$\frac{K_1 L}{1 + K_1 L} k_2 + k_{-2} \frac{1}{1 + K_3 L} = (\tau_{fast})^{-1} \qquad (2)$$

$$k_4 + k_{-4} \frac{K_3 L}{1 + K_1 K_3 L} = (\tau_{slow})^{-1} \qquad (3)$$

In order to test the validity of the hypothesis theoretical curves corresponding to scheme 1 were fitted to the experimental data points. A wider range of ligand concentration was used in the simulations; the equilibrium and kinetic parameters derived therefrom are listed in Table 1. Given the values of the resulting K_{eq}'s and the fact that the binding steps did not make themselves apparent in the kinetic experiments (sampling rate 40 μsec^{-1}), and setting a lower limit for the off-rate constants of the binding steps in the order of 10^4-10^5 sec^{-1}, binding rates in the region of the theoretical diffusion-controlled limit could be calculated. The possibility that rate-enhancing mechanisms are

Table 1

Calculated equilibrium and kinetic parameters for the interaction of compound III with the membrane-bound AChR from T. marmorata[§]

K_1	$6.7 \pm 1 \times 10^5$ M^{-1}
k_2	118 ± 19 sec^{-1}
k_{-2}	150 ± 25 sec^{-1}
K_3	$5 \pm 0.4 \times 10^6$ M^{-1}
k_4	9700 ± 2500 sec^{-1}
k_{-4}	24 ± 0.6 sec^{-1}

[§]The calculated values result from the expansion of the theoretical fit to ligand concentrations not covered in the experiments. The fit employed Marquardt's algorithm and was implemented by J. Chandler.

operative in the case of the pyrene derivative on a membrane-bound target (see ref. 1 for discussion of the topic) cannot be discarded. Diffusion-controlled binding rates have been proposed for antagonist-ligand interactions in vivo (21) Since antagonists do not open AChR-associated ionic channels, their binding rates cannot be assessed with electrophysiological techniques but only inferred, indirectly, by their effect on agonist activity. No direct measurements of the molecular rate constants for antagonist binding have been reported to date.

The present study provided some insight into an additional aspect of antagonist blocking action: A biliganded AChR state was observed at high ligand concentration, a state reached through the successive binding-isomerization steps rate-limited by the second isomerization. Does this imply that only this final state corresponds to the blocked, unresponsive AChR in vivo? A paradox which can be traced back to Jenkinson's (22) observations on the action of curare is the fact that only one antagonist molecule seems to suffice to block the AChR, whereas at least two acetylcholine molecules are almost certainly needed to activate the AChR (see review in ref. 23). Recent voltage-jump relaxation experiments have disclosed that in addition to the competitive block, curare and related typical "competitive" antagonists can hinder the function of AChR-associated channels in a strongly voltage-dependent manner (24,25). Although curare seems to have more selectivity for open channels (24,25) other agents displaying this type of blockage act non-selectively on open or closed channels, as does the local anaesthetic procaine (26,27). Unless an unexpected proportion of AChR-associated channels are spontaneously open in vitro in the absence of agonist (which is very unlikely) and given the lack of resting membrane potential in AChR-rich membranes under equilibrium ionic conditions, the reaction scheme 1 explains satisfactorily both competitive and non-competitive effects of antagonists, without invoking "channel plugging" mechanisms in vitro, simply by con-

sidering that the two sites on the AChR which bind the antagonist (see scheme 1) are non-equivalent. Experiments in which the kinetics of AChR-antagonist interactions are studied in the presence of an agonist are needed to determine which of the steps correspond to each type of blocking mechanism and to further test the "dual blocking hypothesis" in vitro. As is usually the case in relaxation kinetics, one cannot exclude that many other alternative, more complex mechanisms explain the experimental observations equally well. The reaction scheme and interpretation proposed here, however,are probably the simplest to account for the complex behaviour of the relaxation times and the pharmacological observations.

Acknowledgement. Part of this work was supported by a grant from the Deutsche Forschungsgemeinschaft to F.J.B. (Ba 671/1).

References

1. Barrantes, F.J. (1979) Ann. Rev. Biophys. Bioeng. 8, 287-321.
2. Grünhagen, H.H., Iwatsubo, M. and Changeux, J.-P. (1977) Eur. J. Biochem. 80, 225-242.
3. Quast, U., Schimerlik, M. and Raftery, M.A. (1978) Biochem. Biophys. Res. Commun. 81, 955-964.
4. Quast, U., Schimerlik, M. and Raftery, M.A. (1979) Biochemistry 18,1891-1901.
5. Heidmann, T. , Iwatsubo, M. and Changeux, J.-P. (1977) C.R. Acad. Sci. Paris Ser. D 284, 771-774.
6. Heidmann, T. and Changeux, J.-P. (1979) Eur. J. Biochem. 94, 255-279.
7. Heidmann, T. and Changeux, J.-P. (1979) Eur. J. Biochem. 94, 281-296.
8. Neumann, E. and Chang, H.W. (1976) Proc. Nat. Acad. Sci. U.S.A. 73,3994-3998.
9. Bonner, R.F., Barrantes, F.J. and Jovin, T.M. (1976) Nature 263, 429-431.
10. Barrantes, F.J. (1976) Biochim. Biophys. Res. Commun. 72, 479-488.
11. Barrantes, F.J. (1978) J. Mol. Biol. 124, 1-26.
12. Jürs, R., Prinz, H. and Maelicke, A. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 1064-1068.
13. Weber, G., Borris, D., De Robertis, E., Barrantes, F.J., La Torre, J.L. and Carlin, M.C.L. de (1971) Mol. Pharmac. 7, 530-537.
14. Cohen, J.B., Weber, M. and Changeux, J.-P. (1974) Mol. Pharmac. 10, 904-932.
15. Barrantes, F.J., Sakmann, B., Bonner, R., Eibl, H. and Jovin, T.M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3097-3101.
16. Barrantes, F.J., Tan, Y., Stender, W. and Harvey, A. Mol. Pharmac., submitted
17. Weber, M., David-Pfeuty, T. and Changeux, J.-P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3443-3447.
18. Weiland, G., Georgia, B., Wee, V.T., Chignell, C.F. and Taylor, P (1976) Mol. Pharmac. 12, 1091-1105.

19.Eigen, M. and De Maeyer, L. (1963) in Techniques of Organic Chemistry. Fries, S., Lewis, E. and Weissberg, A. eds. 8, 896-1054. New York, Wiley.

20.Loudon, G.M. and Koshland, D.E. (1972) Biochemistry 11, 229-240.

21.Sheridan, R.E. and Lester, H.A. (1977) J. Gen. Physiol. 70, 187-219.

22. Jenkinson, D.H. (1960) J. Physiol. 152, 309-324.

23.Steinbach, J.H. and Stevens, C.F. (1976) in Frog Neurobiology. Llinas, R. and Precht, W. eds. 32-92. Springer, Berlin.

24.Ascher, P., Marty, A. and Neidl, T.O. (1978) J. Physiol. 278, 207-235.

25.Marty, A. (1978) J. Physiol. 278, 237-250.

26.Colquhoun, D., Dreyer, F. and Sheridan,R.F. (1979) J. Physiol.293,247-284.

27.Adams, P.R. (1977) J. Physiol. 268, 291-318.