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INTERACTION OF A FLUORESCENT AGONIST WITH THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR FROM TORPEDO MARMORATA IN THE MILLISECOND TIME RANGE: RESOLUTION OF AN"INTERMEDIATE" CONFORMATIONAL TRANSITION AND EVIDENCE FOR POSITIVE COOPERATIVE EFFECTS.

by

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SUMMARY

Stopped-flow measurements of the rapid kinetics of interaction of the fluorescent agonist Dns-C_6-Cho with the membrane-bound acetylcholine receptor (AChR) were extended to the 0.1 to 1 mM range of agonist concentration. In this domain of concentrations, a fluorescent intensity increase is observed in the milliseconds time range. The signal follows a monoexponential time course and is abolished upon preincubation of the membrane fragments with saturating concentrations of Naja nigricollis α-toxin.

A plot of the rate constant of the signal as a fonction of agonist concentration deviates from linearity: k_{app} first increases in a sigmoid manner (n_H = 1.86) for concentrations up to 300 μM, and then decreases approx. two-fold for concentrations up to 1 mM. A minimal scheme involving a conformational transition between discrete low affinity states of the receptor, with two agonist binding sites per molecule is proposed; the relevance of these states and transition to the physiological mechanism of activation and to the fast and slow desensitisation processes is discussed.

INTRODUCTION

Fast kinetic analysis of the interaction of a fluorescent cholinergic agonist Dns-C_6-Cho with the membrane-bound acetylcholine receptor (AChR) has revealed

ABBREVIATIONS: ACh, acetylcholine; AChR, acetylcholine receptor; Dns-C_6-Cho (1-(5-dimethylamino-naphthalene) sulfonamido) n-hexanoic acid β-(N-trimethyl ammonium bromide) ethyl ester.

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three main relaxation processes (1, 2): 1) a "rapid" relaxation process, which is accounted for by a diffusion controlled binding of the agonist to a fraction of receptor sites preexisting in a high affinity state ($K_D \sim 3$ nM), 2) a "slow" relaxation process in the second time scale, corresponding to the interconversion of the receptor from low to the high affinity state and 3) an "intermediate" relaxation process, which was interpreted, in a first approach, as resulting from the binding of Dns-C_6-Cho to low affinity sites present in the membrane at rest. The amplitude of this intermediate relaxation process reached half of its maximum value for a micromolar concentration of agonist and no plateau value for the apparent rate constant (k_{app}) could be detected in the domain of Dns-C_6-Cho concentration investigated (i.e. up to 50-100 µM). It was nevertheless inferred from the low absolute values of the onward and backward rate constants and from the acceleration of k_{app} by allosteric effectors such as the aminated local anesthetics, that this "intermediate" relaxation process includes a transition towards a third conformational state of the receptor (2).

In this Communication, an extensive and quantitative analysis of the intermediate relaxation process in the high Dns-C_6-Cho concentration range (up to 1 mM) is presented. This analysis was made possible by numerical accumulation of single-shot traces of stopped-flow experiments (up to 10) in a digital recorder, which significantly increases the signal-noise ratio otherwise too low for single shot traces analysis in the high concentration range of agonist. The data are consistant with the presence of an isomerisation of the membrane-bound receptor protein in the millisecond time range and of positive cooperative interactions between at least two agonist binding sites.

MATERIALS AND METHODS

Purified AChR-rich membranes from T. marmorata: AChR-rich membrane fragments were purified from freshly dissected electric tissue from T. marmorata as described in (3), with the modification given in (4) to limit proteolysis, and stored in liquid nitrogen. The AChR sites were assayed with α-^{125}I-bungarotoxin (50-150 Ci/mmole) by column filtration as in (3).

Stopped-flow experiments: Concentrated suspension of AChR-rich membrane fragments was diluted at least 100 fold in Torpedo saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM sodium phosphate buffer pH 7) to a final concentration of approx. 0.2 µM α-toxin binding sites, before rapid mixing with a solution of Dns-C_6-Cho in the same medium, in a Gibson-Durrum stopped-flow apparatus equipped for fluorescence detection. Fluorescence was excited at 290 nm using a 450 W Osram xenon lamp and a grating monochromator, and 90° light emission was monitored with a Hamamatsu R 376 photomultiplier using a high pass filter (540 nm cut), as described previously (1, 2). Single-shot fluorescence signals were digitally stored in a TRACOR NS 570 (12 bits, 1024 points) and the mean signal resulting from digital accumulation of 1-10 single-shot experiments plotted with an X-Y recorder. The traces were fitted by a non-linear iterative regression program (least square fit criterion) using a Numonics planimeter on line with a Nova 3D computer (Centre de Calcul de l'Institut Pasteur) to which were given 100 points/curve.

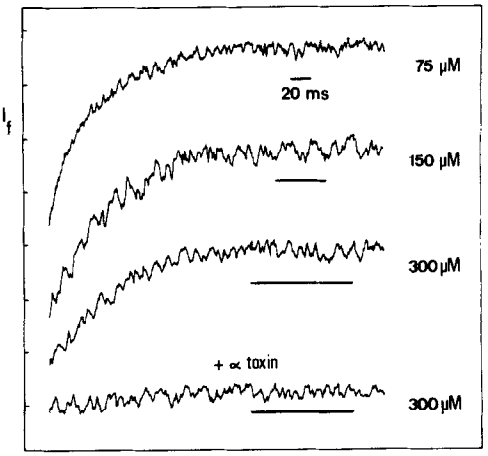


Figure 1: Increases of fluorescence intensity recorded after rapid mixing of AChR-rich membrane fragments with various concentrations of Dns-C_6-Cho in a stopped-flow apparatus. Lower trace: blocking of the fluorescence signal after preincubation of the membrane fragments with N. nigricollis α-toxin.
λ_{ex} = 290 nm, λ_{em} > 540 nm; 1:1 mixing of a suspension of receptor-rich membrane fragments in Torpedo saline solution (0.2 µM α-toxin binding sites) with solutions of Dns-C_6-Cho in the same medium (final concentrations after mixing as indicated in the figure). The traces are the mean of 1-10 single-shot experiments (see Methods). Blocking effect of α-toxin: the AChR-rich membrane fragments (as above) were preincubated for 30 min with an excess of N. nigricollis toxin (1 µM) and the experiment carried out as previously.

Chemicals: Dns-C_6-Cho was a gift of G. Waksman, M.C. Fournié-Zaluski and B. Roques who synthetised it (5). α-^{125}I-labelled bungarotoxin was from NEN. N. nigricollis α-toxin was a gift from Dr. P. Boquet.

RESULTS

Rapid mixing in a stopped-flow apparatus of suspensions of AChR-rich membrane fragments with solutions of Dns-C_6-Cho yields, up to 1 mM, increases of fluorescence intensity which are completely abolished by preincubation of the membrane fragments with saturating levels of Naja nigricollis α-toxin and therefore result from the interaction of Dns-C_6-Cho with the AChR-site (Fig. 1). As already presented (2), at Dns-C_6-Cho concentration lower than ∿ 20 µM a slow relaxation process corresponding to a conformational transition in the second time range towards a high affinity state of the AChR is observed but can be resolved from more rapid signals. On the other hand, at Dns-C_6-Cho concentration higher than ∿ 20 µM, the traces recorded are fitted by single exponentials and their rate constant (k_{app}) determined. As illustrated in Fig. 2, k_{app} increases as a function of Dns-C_6-Cho concentration in a sigmoid manner and reaches a maximum at approx. 300 µM Dns-C_6-Cho. Beyond this concentration, k_{app} decreases approx. two folds up to 1 mM; A replot of these data in the double logarithmic coordinates of Hill (log (k_{app}/k_{app}max. - k_{app}) versus log (Dns-C_6-Cho)) gives, for Dns-C_6-Cho concentrations up to 200 µM, a straight line with a slope equal to 1.86 ± 0.05 (see Fig. 2 insert).

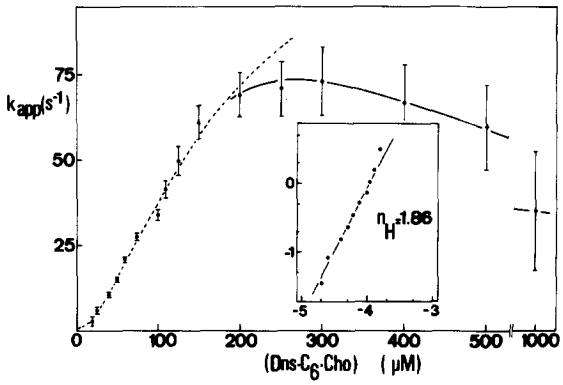


Figure 2: Plot as a function of Dns-C_6-Cho concentration of the apparent first order rate constant k_{app}.
Same experimental conditions as in Fig. 1; the dotted line is the best fit (least-square fit criterion) of the data up to 200 μM agonist concentration, according to equation :

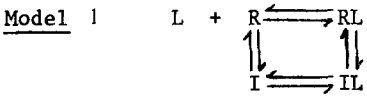
$$k_{app} = k_2 \frac{(Dns\text{-}C_6\text{-}cho)^2}{K^2+2K(Dns\text{-}C_6\text{-}Cho)+(Dns\text{-}C_6\text{-}Cho)^2} + k_{-2}$$

This equation is the derivation of k_{app} from model (2) (see Results) where $K = k_{-1}/k_1$, assuming that binding occurs rapidly as compared to isomerisation. The best fit is obtained for the following values: k_{-1}/k_1 : 119 ± 10 μM, k_2 = 180 ± 20 s^{-1} and k_{-2} = 1.2 ± 0.3 s^{-1} (k_{-2} was derived from the values of k_{-1}/k_1, k_2 and the apparent dissociation constant for the stabilisation of the I state, see 2).

Insert: Hill plot of the data: log ($k_{app}/k_{app}max - k_{app}$) in ordinates, and log (Dns-C_6-Cho) in abscissa.

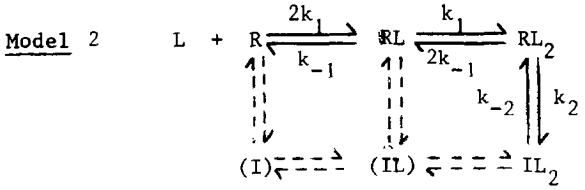
The complex dependance of k_{app} with Dns-C_6-Cho concentration can no longer be accounted for by a simple bimolecular binding reaction since, if it was the case, k_{app} should increase linearly with the agonist concentration. On the other hand, the existence of limiting values for k_{app} can be interpreted on the basis of a conformational transition which accompanies binding of the ligand, according to:

Model 1



This simple mechanism however does not account for the sigmoid shape of the concentration dependance for k_{app} as a function of Dns-C_6-Cho (Fig. 2). The next minimum model is:

Model 2



Model 2 (solid arrows) is a simplified version of the concerted two-state model for allosteric transitions (6, 7; see also 8-12) where it is assumed that 1) the isomerisation between R and I, and RL and IL are much slower than between RL_2 and IL_2, and 2) that one molecule of ACh receptor binds two molecules of Dns-C_6-Cho with identical intrinsic affinities. As illustrated in Fig. 2 (see dotted line), this model accounts satisfactorily for the data for concentrations of Dns-C_6-Cho up to 200 μM, and in particular for the sigmoid dependence of k_{app}. The best fit of the data is obtained with 119 ± 10 μM for the intrinsic dissociation constant and 180 ± 20 s^{-1} and 1.2 ± 0.3 s^{-1} for k_2 and k_{-2} respectively. Important deviations are however observed at higher agonist concentrations. In particular, model 2 gives plateau values for k_{app} which are at least two-fold higher than the observed values, moreover it does not account for the observed decrease of k_{app} in the millimolar concentration range.

Several interpretations for the observed deviations are plausible. All of them require the binding of additional Dns-C_6-Cho molecules to the membrane-bound AChR, which could result in a decrease of the rate of isomerisation between the R and I states. Dns-C_6-Cho might bind at the level of a third acetylcholine receptor-site present on the receptor protein. However, it is well established that the α chain of the AChR carries the α-toxin labeled AChR-site (13, 14), and the presently available data on the quaternary structure of the purified (15, 16) and membrane-bound (17) receptor, indicate that there are only two α-chains per receptor molecule. On the other hand, at these high concencentrations, Dns-C_6-Cho might also bind to the site for non-competitive blockers (18, review in 19) distinct from the AChR site and carried, at least in part, by the δ subunit of the receptor (20, 4). This possibility appears plausible since it has been shown that at high concentration, Dns-C_6-Cho exerts a "local anesthetic-like" effect on the permeability response, both in vivo by following membrane potential with E. electricus electroplaque (2, 5, 21) and in vitro by following ^{22}Na efflux from AChR-rich microsacs from T. marmorata (2).

DISCUSSION

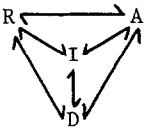
The data presented in this paper provide evidence for the occurrence of a transient low affinity state with half-life time in the millisecond time scale and for a cooperative interaction of the fluorescent agonist Dns-C_6-Cho with the membrane-bound AChR.

Cooperative binding of radioactive cholinergic agonists have been observed with the membrane-bound (18, 22-26) and the detergent extracted (27-29) AChR, under equilibrium conditions i.e. with a high affinity state of the receptor. The present study demonstrates that cooperative binding also occurs upon rapid addition of the agonist with a low affinity state of the membrane-bound receptor. These results are consistent with the occurrence of at least two AChR-sites (13, 14) per AChR oligomer.

They are also consistent with the sigmoid shape of the dose-response curves for ionic conductance, observed both in vivo (8, 30-34; ref. in 35) and in vitro (12, 36, 37).

The physiological interpretation of the rapid kinetic data cannot be ascertained since ion fluxes yet are not available with Dns-C_6-Cho in the millisecond time range. Preliminary results however indicate (see also 2) that this agonist exerts its pharmacological action in a domain of concentrations close to that of the physiological transmitter acetylcholine. In fact, the dissociation constant of Dns-C_6-Cho for the R state is, according to Model 2, found close to the dissociation constant of ACh for the physiological process of "activation": approx. 30 μM from in vivo electrophysiological experiments (8, 34, 38, 39; ref. in 35) and 70-150 μM from in vitro rapid measurements of radioactive ion fluxes (40, see also 12, 37 for carbamylcholine). The "intermediate" transition analyzed in this Report, leads to a state (I) with a dissociation constant close to 1 μM and takes place in a time range (10-100 msec) at least one order of magnitude slower than the activation reaction. It, therefore, cannot be identified to the opening of the ion channel by the agonist i.e. the transition of the receptor from the resting R state to its active A conformation. However, this still unresolved R$\rightleftharpoons$A reaction should be included in what we have referred to as the "binding" of the agonist to the R state.

On the other hand, the rate constant of this "intermediate" process is too fast to be associated with the classical process of pharmacological "desensitization", which develops in the second(s) time range both in vivo and in vitro and is accounted for by the slow transition of the AChR toward its high affinity state (2, review in 19). Interestingly, recent electrophysiological experiments following single-channel conductance (38, see also 41) have revealed a fast component in channel inactivation in the ms time range superimposed on the slow component in the second time range of desensitization. In the presence of 20 μM ACh this fast component has an apparent rate constant of 2-5 s^{-1} which is close to that of the fluorescence signal obtained at the same concentration with Dns-C_6-Cho (3 s^{-1} see Fig. 2). Finally, this transition could also correspond to that monitored upon agonist addition with AChR-rich membrane fragments extrinsically labeled with the fluorescent local anesthetic quinacrine, which develops in the milliseconds time range with a closely related agonist concentration dependence (42). Accordingly, in agreement with the data and interpretations of Neubig and Cohen (37), a minimal scheme of the conformational transition of the membrane-bound AChR would include four states,



resting R, active A, intermediate I and desensitized D in reversible equilibrium. The

ion channel would be open only in the A state but the four states would differ by their affinity for cholinergic ligands and non-competitive blockers. Yet, only the transitions to the I and D states have been demonstrated by rapid binding kinetics.

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