Ligand-specific state transitions of the membrane-bound acetylcholine receptor

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We have developed a simple, direct and time-resolved method to monitor ligand-induced changes in agonist affinity of the membrane-bound acetylcholine receptor. The assay is based on the quenching of fluorescence of NBD-5-acylcholine observed upon binding of this cholinergic agonist to the receptor. Under conditions of partial saturation with the fluorescent agonist, agonists and local anesthetics but not antagonists can induce an increase in affinity of the receptor for NBD-5-acylcholine. The effect is not observed with receptor fully saturated with the fluorescent agonist. The half-life of the observed change in affinity is independent of the nature of the agonist or local anesthetic applied ($t_{1/2}$ ~60 s at 22°C). We conclude that the same state transition of the receptor can be induced by two groups of cholinergic ligands that are assumed to be non-competitive with each other and to have distinctly different modes of action. The time course of the transition is reminiscent of the slow process of desensitization observed in vivo.

Acetylcholine receptor *Agonist affinity* *State transition* *Local anesthetic* *Fluorescence kinetics* *Ligand binding* Torpedo marmorata

1. INTRODUCTION

A convenient approach to analysis of the electrophysiological data on muscle excitation in more molecular terms is the assumption of 3 states of the acetylcholine receptor integral cation channel [1,2]; an active (ion conducting) and two inactive (the resting and the desensitized) states. Acetylcholine and its agonists are assumed to convert the resting into the active state and to induce conversion of the active to the desensitized state. Antagonists which bind mutually exclusively with agonists to the receptor are assumed to interact only with the resting state. Local anesthetics which bind to separate sites [3] but, in addition, can interfere with the binding of other ligands to the receptor are also assumed to cause state transitions of the agonist activated channel [4].

To probe further into the molecular basis of the primary events of muscle excitation, biochemical correlates to the established electrophysiological phenomena are required. We have approached this problem by studying with rapid mixing techniques the interaction of affinity-purified acetylcholine receptor with the full agonist NBD-5-acylcholine [5–7]. Expanding this work to the receptor in its natural membrane environment, we demonstrate here ligand-specific state transitions of the acetylcholine receptor. We show that agonists and local anesthetics but not antagonists can induce a state transition of the receptor that by its time course correlates with the slow process of desensitization observed with intact muscle endplates and reconstituted systems.

2. EXPERIMENTAL

Acetylcholine receptor-rich membrane vesicles from *Torpedo marmorata* were prepared as in [8], with the concentration of NBD-5-acylcholine binding sites obtained by equilibrium binding studies [8,9]. Fluorescence kinetic and equilibrium binding studies were performed as in [6,7].

The membrane fragments (approx. 10^{-5} M in α-toxin binding sites) were stored at 4°C in 10 mM sodium phosphate, 10 mM NaN_3, 5 mM EDTA

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(pH 7.4) and used within 6 days of their preparation. For the actual experiments, the membrane fragments were diluted (100-fold or more) into standard buffer (100 mM NaCl, 4 mM $CaCl_2$ 2 mM $MgCl_2$, 10 mM Pipes; pH 6.8) and preincubated with Tetram for 30 min at 4°C. Subsequently NBD-5-acylcholine in standard buffer was added in a small volume (<0.5% of the total) and the mixture was further incubated at room temperature for at least 15 min. These solutions were then employed in the equilibrium binding and stopped-flow experiments detailed in the figure legends.

The concentration of NBD-5-acylcholine binding sites and the degree of receptor saturation were calculated assuming model 1a of authors in [7] and a K_D value of 17 nM for both classes of sites [9].

3. RESULTS AND DISCUSSION

In the experiments described below, the concentration of receptor and NBD-5-acylcholine was kept constant throughout. Since receptor was preequilibrated with fluorescent ligand, the initial level of fluorescence (origin in the plots) related to the relative amounts of bound and free ligand in the reaction mixture. Since binding of the fluorescent ligand to the receptor is accompanied by fluorescence quenching, the increase in fluorescence in the course of the experiments correlated with a decrease in the concentration of bound NBD-5-acylcholine and vice versa.

Fig.1 shows the changes in fluorescence *vs* time observed after rapid mixing of a preequilibrated mixture of membrane vesicles and NBD-5-acylcholine with either d-tubocurarine (left) or acetylcholine (right). Addition of d-tubocurarine caused an increase in fluorescence, the equilibrium value (determined at 100 s) of which agreed with the amount of NBD-5-acylcholine competed from the receptor by the antagonist [9]. Addition of acetylcholine caused more complicated effects: At high concentrations of acetylcholine (traces 3–5), i.e., when most or all of the receptor is occupied by

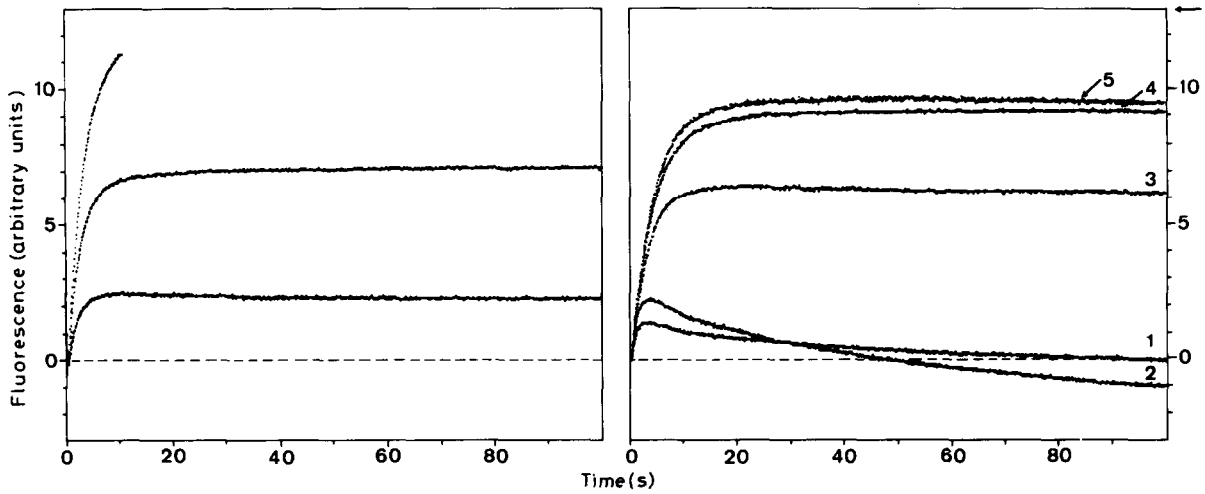

Fig.1. Kinetics of competition of non-fluorescent cholinergic ligands with preformed complexes of AChR-rich membrane fragments from *Torpedo marmorata* and NBD-5-acylcholine. (Left) Membrane fragments, approx. 0.08 μM in NBD-5-acylcholine binding sites, were preequilibrated with 0.045 μM fluorescent ligand resulting in approx. 40% saturation of the binding sites [9]. Equal volumes of this reaction mixture and of d-tubocurarine, both in standard buffer (100 mM NaCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$ 10 mM Pipes; pH 6.8) were rapidly mixed in a Durrum D100 stopped-flow spectrofluorimeter and the fluorescence was recorded as a function of time. The concentrations of d-tubocurarine after mixing were (from below) 0.01, 0.05 and 0.5 μM, respectively. The arrow at the right indicates the final level of fluorescence achieved with the highest concentration. (Right) Under otherwise identical experimental conditions acetylcholine instead of tubocurarine was employed in the stopped-flow experiments. The concentrations of acetylcholine after mixing were (1) 0.015 μM, (2) 0.03 μM, (3) 0.3 μM, (4) 3 μM and (5) 30 μM. Rate constant of the fluorescence decay observed in traces 1 and 2 and determined as in [27]: $k_{app} = 0.0175\ s^{-1}$. (The traces shown are each the average of 4 experiments performed successively under otherwise identical conditions.)

acetylcholine at equilibrium, the pattern resembled that observed with d-tubocurarine. At lower concentration of acetylcholine (traces 1,2), however, an initial increase in fluorescence was followed by an additional decrease in fluorescence not observed with d-tubocurarine. Given the appropriate conditions (trace 2), the final level of fluorescence was even lower than that prior to mixing. We interpret this observation as the result of two parallel processes, the usual ligand competition (as in the case of d-tubocurarine) and a state transition induced by the binding of acetylcholine. Since this state transition is observed as a decrease in fluorescence, it is accompanied by an increase in fluorescent ligand bound.

One simple explanation for this effect is to assume that binding of acetylcholine to one of the two sites [2,6] of previously unoccupied receptor increases the affinity for agonists of the second of the two sites. This then leads to preferential (and additional) binding of NBD-5-acylcholine to these sites thereby causing the observed decrease in total fluorescence. The proposed explanation is equivalent with the assumptions of positively cooperative interactions between the two agonist sites at the receptor [4,8] and additivity of binding of agonists. It also rationalizes the fact that the effect is observed only with partially saturated receptor.

Similar experiments were performed with a variety of other non-fluorescent agonists and antagonists. Consistent with the observations described above, only agonists and the metaphilic antagonist hexamethonium [10,11] caused an increase in the affinity of binding for NBD-5-acylcholine after the initial phase of competition. From the range of agonist concentrations required to induce comparable effects as shown in fig.1, a strong correlation with the apparent dissociation constants of these ligands was observed. For instance, carbamoylcholine showed its maximal effect at 0.4 μM and acetylcholine at 0.024 μM. In all experiments, i.e., independent of the specific agonist applied, the half-life of the slow isomerisation step was approx. 60 s at 22°C. This half-life is similar to that of the slow process of desensitization observed in electrophysiological experiments with frog endplates [12,13], in ion-flux experiments with *Torpedo* membrane vesicles [14] and in patch-clamp studies with reconstituted *Torpedo* receptor [15].

As is apparent from the initial phase of competition preceding the increase in affinity for the fluorescent ligand, the rate of dissociation of bound fluorescent ligand was faster than the rate of the state transition. Thus, we do not know at present whether a preexisting equilibrium between two or more affinity states of the non-liganded receptor is shifted in the presence of agonist in the direction of the high-affinity state(s) [16,17] or whether particular forms of the liganded receptor are responsible for the observed high affinity of binding for agonists [2,7]. It is clear, however, that the size of the effect correlates with the probability of formation of mixed diliganded complexes of the receptor, i.e., competing agonist : receptor : NBD-5-acylcholine.

The concentration dependence of the observed changes in total fluorescence of the reaction mixture was also studied under equilibrium conditions. For this purpose, aliquots of competing ligand were injected into a cuvette containing membrane vesicles and a non-saturating concentration of NBD-5-

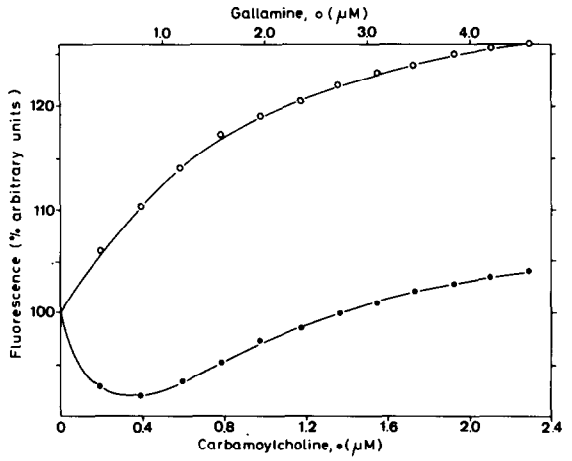

Fig.2. Competition at equilibrium of non-fluorescent cholinergic ligands with preformed complexes of membrane-bound acetylcholine receptor and NBD-5-acylcholine. Membrane fragments, approx. 0.05 μM in NBD-5-acylcholine binding sites, were preequilibrated with 0.037 μM NBD-5-acylcholine resulting in approx. 49% saturation of the receptor's ligand binding sites [9]. Into 2 ml of this solution in a fluorescence cuvette, gallamine (○) or carbamoylcholine (●) was added in steps (8 μl per step) employing a Hamilton Microlab P titrator [6]. Following each application, the fluorescence (λ_{ex} = 480 nm, 1 nm bandwidth, emission recorded through a Dietrich 520 nm cut-on filter) was recorded for 500 s.

acylcholine. After equilibrium was achieved, the fluorescence was recorded followed by the addition of the next aliquot of competing ligand. As exemplified in fig.2 for the agonist carbamoylcholine and the antagonist gallamine, only agonists showed a biphasic pattern under these conditions. At low concentration of agonist, the increase in affinity of binding for NBD-5-acylcholine outweighed the competing effect and a net decrease in fluorescence (increase in fluorescent ligand bound) was observed. With increasing concentration of agonist the competing effect became increasingly dominant resulting in complete recovery of fluorecence (complete removal of the bound fluorescent ligand) at sufficiently high agonist concentration. In contrast, antagonists did not cause any decrease in total fluorescence and, thus, did not induce this state transition. (A close inspection of the experiment with d-tubocurarine (fig.1, lowest trace) indicates, however, that this ligand may induce the observed state transition to a very limited extent. This may relate to a more complicated binding pattern [18] and direct channel blocking properties [19] of this ligand.)

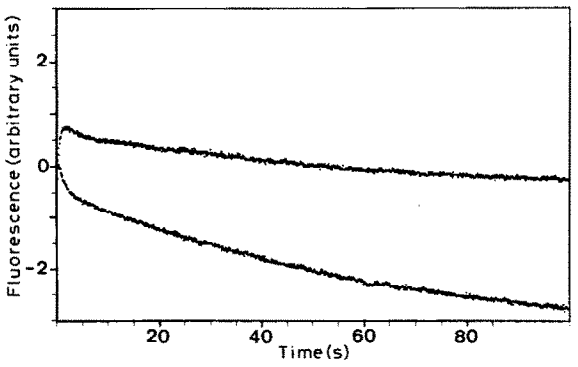

Fig.3. Kinetics of interaction of local anesthetics with preformed complexes of AChR-rich membrane fragments and NBD-5-acylcholine. The concentrations of receptor and NBD-5-acylcholine were identical to those indicated in fig.1. Equal volumes of this reaction mixture and of either procaine (upper trace) or dibucaine (lower trace) were rapidly mixed and the fluorescence was recorded as a function of time. Each trace shown represents the average of 4 experiments performed successively under otherwise identical conditions. The concentrations after mixing were 500 μm for procaine and 10 μM for dibucaine. Rate constants of the fluorescence decay obtained as in [27]: procaine, $k_{app} = 0.010\,s^{-1}$; dibucaine, $k_{app} = 0.015\,s^{-1}$.

The observed state transition is also induced by local anesthetics as shown in fig.3. The simple effect caused by the rather lipophilic local anesthetic dibucaine (fig.3, lower trace) appeared consistent with its action as a mainly non-competitive blocking agent of the receptor [20]. The kinetic pattern almost exclusively showed a decrease in fluorescence, probably biphasic, but practically no initial phase of competition as was observed with agonists (fig.1). In contrast, the more hydrophilic local anesthetic procaine showed a phase of competition (fluorescence increase) followed by a phase of state transition (fluorescence decrease). In addition, procaine was much less powerful than dibucaine in inducing the state transition.

4. CONCLUSIONS

Our studies extend previous findings of an agonist-induced transition to a state of high affinity [11,16,17,21–23] and of conformational changes in the course of ligand binding [24,25] of the acetylcholine receptor. They establish the reproducibility and significance of an effect first observed by authors in [16] but not studied in further detail by them. Employing equilibrium binding and kinetic studies and several representative ligands of the receptor we can now draw the following conclusions on the observed state transition of the acetylcholine receptor:

(i) The transition is observed with membrane-bound but not with purified receptor [6,7] and, thus, requires the presence of membrane components or the existence of a particular conformation of the receptor lost during solubilization and purification;

(ii) The transition is induced only by agonists and local anesthetics, i.e., by ligands known to affect the properties of the receptor-integrated channel but not by antagonists;

(iii) The time course of the transition conforms to the slow process of desensitization.

Our studies do not suffice to establish the molecular basis of the isomerization process. In the case of agonists it is tempting to assume that the transition is caused by the same mechanism underlying the positive cooperativity of agonist binding sites [8]. However, since local anesthetics are assumed to bind to separate sites at the receptor–channel complex, the latter ligands would then have to in-

duce the transition from a different site and, thus, by a different molecular mechanism. Another possibility not excluded by the present study is to assume a common regulatory site structurally distinct from the agonist binding sites for both cholinergic agonists and local anesthetics at the receptor–channel complex.

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