

# Desensitization is a property of the cholinergic binding region of the nicotinic acetylcholine receptor, not of the receptor-integral ion channel

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The reversible acetylcholine esterase inhibitor (-)-physostigmine (eserine) is the prototype of a new class of nicotinic acetylcholine receptor (nAChR) activating ligands: it induces cation fluxes into nAChR-rich membrane vesicles from *Torpedo marmorata* electric tissue even under conditions of antagonist blocked acetylcholine binding sites (Okonjo, Kuhlmann, Maelicke, Neuron, in press). This suggests that eserine exerts its channel-activating property via binding sites at the nAChR separate from those of the natural transmitter. We now report that eserine can activate the channel even when the receptor has been preincubated (desensitized) with elevated concentrations of acetylcholine. Thus the conformational state of the receptor corresponding to desensitization is confined to the transmitter binding region, leaving the channel fully activatable - albeit only from other than the transmitter binding site(s).

Nicotinic acetylcholine receptor; Acetylcholine-gated cation channel; Desensitization; Ion flux; Carbamate; Physostigmine; Eserine; Anticholinesterase

## 1. INTRODUCTION

In the prolonged presence of agonist, a slow transition of the nicotinic acetylcholine receptor (nAChR) to one or more inactive, and only slowly reactivatable states takes place [1-4]. The rate and extent of this 'desensitization' depend on the nature and concentration of agonist applied, and there exists a large body of experimental evidence suggesting that desensitization is related to an increase in affinity of the nAChR for agonist [5-9]. There is evidence that reaction of the receptor with at least two molecules of agonist is required to induce channel activation and desensitization [4,10], yet that channel activation is not a prerequisite for desensitization [11]. Noncompetitive blockers such as local anesthetics increase the fraction of receptor desensitized by submaximal doses of agonist [12].

Agonist-induced ion flux into closed membrane vesicles from *Torpedo* electric organ is characterized by kinetic phases resembling those of nAChR activation and desensitization: a rapid phase of flux onset is followed by at least two phases of flux decrease [8,13,14]. The time course of flux decrease correlates with the increase in affinity of the receptor for agonist determined with the same preparation. Thus, ion flux studies expose major properties of the process of desensitization.

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*Abbreviations:* ACh, acetylcholine; AChE, acetylcholine esterase; nAChR, nicotinic acetylcholine receptor

By means of rapid ion flux studies, we have recently found [15] that the reversible acetylcholine esterase inhibitor (-)-physostigmine (eserine) is capable of inducing cation fluxes into nAChR-rich *Torpedo* membrane vesicles even when the binding sites for acetylcholine at the nAChR are blocked by saturating concentrations of antagonist such as D-tubocurarine,  $\alpha$ -bungarotoxin or the monoclonal antibody WF6. Since eserine thus must exert its channel-activating effect from site(s) separate from the transmitter binding sites, it was interesting to analyse whether it can also induce ion flux into membrane fragments preincubated (desensitized) with elevated concentrations of acetylcholine.

## 2. MATERIALS AND METHODS

nAChR-rich membrane vesicles were prepared according to Duguid and Raftery [16], with the minor modification described by Reinhardt et al. [17]. Following sucrose gradient fractionation, the fractions with the highest concentration of nAChR were pooled, diluted with a 10-fold excess of ice-cold distilled water and centrifuged for 30 min at 18 000 rpm in a SS-34 rotor. The pellet was resuspended in 300 mM NaCl, 10 mM Hepes, pH 7.0, and stored at  $-80^{\circ}\text{C}$ . The receptor concentration of the suspension was generally of the order of 17-20  $\mu\text{M}$ , in terms of ACh binding sites, at a protein concentration of 10-17 mg/ml.

Ion flux studies were performed according to Moore and Raftery [18] except that 1,3,6,8-pyrene tetrasulfonate was used as fluorescent dye, and  $\text{Cs}^+$  was used as heavy metal quencher instead of  $\text{Tl}^+$  [19]. Loading of the membrane vesicles with dye was achieved by three cycles of freezing and thawing. Excess dye was removed by passage through a column of Sephadex G25 (coarse) equilibrated with 'Na-buffer' (300 mM NaCl, 10 mM Hepes, pH 7.0), the elution time being approx. 15 min. The eluate was then made 50  $\mu\text{M}$  in the esterase blocker tetram. The receptor concentration of the vesicle suspension, after passage through the column, was of the order of 1  $\mu\text{M}$  ACh binding sites.

Aliquots of the vesicle suspension were rapidly mixed in a HighTech SF-51 MX stopped-flow fluorimeter with equal volumes of 'Cs-buffer' (300 mM CsCl, 10 mM Hepes, pH 7.0) with or without activating ligand. Excitation of fluorescence was achieved by a 240 W xenon/halogen lamp. The light was passed through a Schott UG 11-UV broad-band filter before reaching the cuvet. A Schott KV 399 filter, placed between cuvet and photomultiplier, was employed to absorb any exciting light reaching the photomultiplier pathway. A/D conversion and collection of signals were achieved by a personal computer equipped with a Sorex Modular IV card. The reported kinetic traces were each averaged from at least five independent experiments.

Ion flux studies were performed at 19.6°C within 90 min after loading of the vesicles with dye. Preincubation with ACh was performed with dye-loaded vesicles for 15 min; preincubation with neurotoxin was performed overnight prior to loading the vesicles with fluorescent dye. All experiments were performed in the presence of the esterase blocker tetraam which itself did not show any activity towards the nAChR.

### 3. RESULTS

Fig. 1 exemplifies the channel-activating action of eserine under conditions of antagonist-blocked cholinergic sites. The two upper traces were obtained after dye-loaded *Torpedo* membrane vesicles, preincubated with saturating concentrations of  $\alpha$ -bungarotoxin ( $\alpha$ BTX), were rapidly mixed with Cs-buffer in the

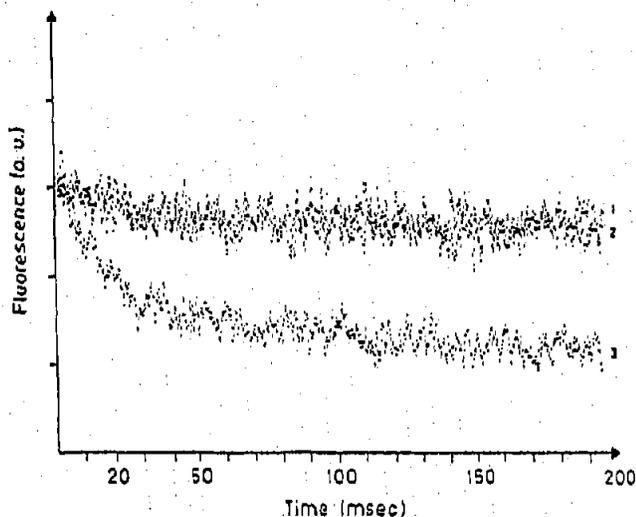


Fig. 1. Eserine-stimulated  $\text{Cs}^+$  influx into membrane vesicles from *Torpedo marmorata* electric organ. (Upper traces) Membrane vesicles (10 nmol of ACh binding sites) suspended in 'Na-buffer' (300 mM NaCl, 10 mM Hepes, pH 7.0) were pretreated overnight at 4°C with 100 nmol of  $\alpha$ -bungarotoxin, loaded with 1,3,6,8-pyrene tetrasulfonate and washed as described in section 2. The vesicle suspension was then rapidly mixed with an equal volume of acetylcholine in 'Cs-buffer' (300 mM CsCl, 10 mM Hepes, pH 7.0). The ACh concentration after mixing was 20  $\mu\text{M}$ . The change in fluorescence (arbitrary units) was recorded as a function of time (ms). The observed slow decrease in fluorescence (trace 2) is identical (within the range of experimental error) to the spontaneous equilibration of  $\text{Cs}^+$  between extracellular space and cytosol in the absence of channel-activating ligands (trace 1, 'leakage kinetics'). (Lower trace) Same experimental conditions as above except that acetylcholine was replaced by eserine (200  $\mu\text{M}$  after mixing). Each trace represents the average of 5 experiments.

absence (trace 3) or presence (trace 2) of 40  $\mu\text{M}$  acetylcholine. Trace 1 represents the 'leakage kinetics' of the vesicle preparation; trace 2, which is indistinguishable from trace 1 within the range of experimental error, is evidence of practically complete blockade of the cholinergic sites by  $\alpha$ BTX. In contrast, when the Cs-buffer was supplemented with 400  $\mu\text{M}$  eserine instead of acetylcholine, rapid mixing with membrane vesicles pretreated with  $\alpha$ BTX resulted in strong and rapid quenching of the fluorescence, demonstrating that eserine-induced  $\text{Cs}^+$ -influx can take place even under conditions of blocked cholinergic sites.

In the second set of experiments, exemplified in Fig. 2, the dye-loaded membrane vesicles were pretreated with an overdose of the natural transmitter prior to rapid mixing with Cs-buffer. Under these conditions of ACh-desensitized nAChR only leakage kinetics were observed independent of whether the Cs-buffer contained acetylcholine or not (traces 1 and 2 in Fig. 2). In contrast, when the ACh-preincubated membrane vesicles were rapidly mixed with Cs-buffer containing eserine, 'flux kinetics' characterized by strong quenching of fluorescence were observed. The total amplitude of fluorescence quenching achieved by the concen-

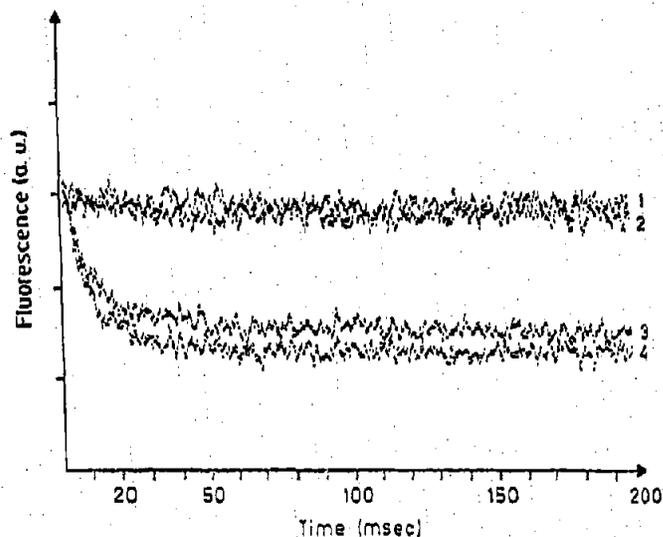


Fig. 2. Eserine-stimulated  $\text{Cs}^+$  influx into *Torpedo* membrane vesicles containing desensitized nAChR. (Upper traces) Dye-loaded membrane vesicles were incubated for 15 min with 200  $\mu\text{M}$  acetylcholine followed by rapid mixing with an equal volume of Cs-buffer with or without 100  $\mu\text{M}$  acetylcholine. Only the slow kinetics of spontaneous  $\text{Cs}^+$  equilibration were observed (traces 1 and 2) suggesting that in the ACh-preincubated sample the majority of channels remained closed due to desensitization of the nAChR. (Trace 3) ACh-pretreated membrane vesicles were rapidly mixed with 1.84 mM eserine (instead of 100  $\mu\text{M}$  ACh) dissolved in Cs-buffer. (Trace 4) Same experimental conditions except that the membrane vesicles were not pretreated with acetylcholine. The observed rapid kinetics of  $\text{Cs}^+$  influx (trace 3) suggest that eserine activated the nAChR channel even though the nAChR was 'desensitized' by acetylcholine.

tration of eserine applied was only slightly smaller (trace 3) than with membrane vesicles that were not pretreated with ACh (trace 4). We do not know whether the decrease in total amplitude is due to limited competition between acetylcholine and the carbamate or whether the conformational change of the nAChR accompanying desensitization by acetylcholine also affects, albeit to a very limited extent, the eserine binding site(s).

#### 4. CONCLUSIONS

The data of Fig. 1 clearly demonstrate that the carbamate (-)-physostigmine (eserine) is capable of activating the cation channel of the nAChR from *Torpedo marmorata* even under conditions of antagonist-blocked cholinergic sites. Neurotoxins (e.g.  $\alpha$ BTX, this study) and competing antibodies (e.g. WF6, see [15]) are particularly well suited for these experiments as the half-lives of their complexes with the receptor are of the order of many hours [20,21]. In contrast, the complexes of the receptor with acetylcholine (and its low molecular weight agonists and antagonists) have half-lives in the subsecond to second time range [5,7,20,22]. It may therefore be argued that the channel-activating effect of eserine under the experimental conditions of Fig. 2 could be due to displacement of acetylcholine (followed by activation from the transmitter binding sites) rather than to activation from independent sites. In view of the similarity of the results depicted in Figs 1 and 2 this appears rather unlikely. Furthermore, the data of Fig. 2 clearly show that preincubation with ACh rendered the channel 'inactivatable' by ACh and its agonists. Consequently, displacement of ACh by agonist, should it indeed occur in appreciable amounts, does not suffice to render the channel activatable again, i.e. to remove desensitization. Thus, the mere fact of activation by eserine of the ACh-desensitized nAChR (Fig. 2) is evidence for an action of eserine via separate binding site(s). It is very probable though that under the conditions of desensitization, such as those employed in the experiments of Fig. 2, the cholinergic sites remain occupied by agonist, as the half-lives of receptor complexes with ACh (and its low molecular weight agonists) increase by orders of magnitude in the course of desensitization [5,8,13,14,22].

In summary, our data show that the desensitized state of the nAChR is not associated with an 'inactivatable channel' [9] but rather that only the pathway of activation via the cholinergic sites is inhibited. Consequently,

the conformational transition accompanying desensitization by ACh (and its agonists) does not involve the receptor-integral ion channel and hence is confined to only the cholinergic binding region. This region has recently been shown to be a rather large surface region of the receptor's  $\alpha$ -subunit formed by at least two discontinuous sequence segments [23].

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