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A second pathway of activation of the *Torpedo* acetylcholine receptor channel

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We have studied the interaction of the reversible acetylcholine esterase inhibitor (−)physostigmine (D-eserine) with the nicotinic acetylcholine receptor (nAChR) from *Torpedo marmorata* electric tissue by means of ligand-induced ion flux into nAChR-rich membrane vesicles and of equilibrium binding. We find that (−)physostigmine induces cation flux (and also binds to the receptor) even in the presence of saturating concentrations of antagonists of acetylcholine, such as D-tubocurarine, α-bungarotoxin or antibody WF6. The direct action on the acetylcholine receptor is not affected by removal of the methylcarbamate function from the drug and thus is not due to carbamylation of the receptor. Antibodies FK1 and benzoquinonium antagonize channel activation (and binding) of eserine, suggesting that the eserine binding site(s) is separate from, but adjacent to, the acetylcholine binding site at the receptor. In addition to the channel activating site(s) with an affinity of binding in the 50 μM range, there exists a further class of low-affinity ($K_d \sim$ mM) sites from which eserine acts as a direct blocker of the acetylcholine-activated channel. Our results suggest the existence of a second pathway of activation of the nAChR channel.

The nicotinic acetylcholine receptor (nAChR) from muscle, brain and electric tissue is a ligand-gated cation channel [1 – 3]. Binding of acetylcholine, or its agonists, induces transient openings of the channel. Antagonists compete with acetylcholine and its agonists in binding to the receptor thereby blocking the channel-activating action of agonists. Other ligands (non-competitive blockers, direct channel blockers) modulate the agonist-activated channel by binding to separate sites [1, 3].

Physostigmine (eserine) is a slowly reversible inhibitor of acetylcholine esterase which acts by carbamylation of the active serine residue within the 'esteratic site' of the enzyme [4]. The carbamylated enzyme intermediate undergoes hydrolysis more slowly than the acetylated intermediate (in the case of acetylcholine hydrolysis), resulting in a slowly reversible inhibition of the enzyme. Eserine exerts both peripheral and central cholinomimetic actions. In contrast to the action of other anticholinesterases such as the organophosphates, however, eserine and other carbamates have quite diverse actions on the pre- and postsynaptic region of neuromuscular junctions [5 – 7] suggesting that carbamylation of the enzyme might not be their only mode of action. Recently, the laboratory of Albuquerque has provided electrophysiological evidence in favour of a direct action of carbamates at the nAChR [8 – 12]. (−)Physostigmine (eserine), at concentrations below 1 μM, induced single channel currents with amplitudes and open time characteristics typical for the nAChR channel [8 – 13]. This concentration is 5 – 10-fold below the IC_{50} of acetylcholine esterase inhibition, suggesting that the direct action on the receptor might at least equal in importance the action on the enzyme. At higher concentrations, eserine exhibits noncompetitive antagonism with respect to cholinergic agonists, accompanied by rapid opening and closing events (flickering) of the activated channels [8 – 10, 14].

Vesicular membrane fragments from *Torpedo* electric tissue permit the direct measurement of both ligand binding to the nAChR and ligand-induced ion flux through the nAChR-integral cation channel. To remain within the time range of excitatory events at the neuromuscular junction, ion flux is best studied by time-resolved fluorimetry employing rapid-mixing devices [15 – 18]. For this purpose, we prepared sucrose-gradient-purified membrane vesicles from *T. marmorata* electric tissue [19], loaded them with 1,3,6,8-pyrene tetrasulfonate [20], and monitored the quenching of fluorescence induced by influx of Cs^+ [21] after rapid mixing, in a stopped-flow apparatus, of dye-loaded membrane vesicles with a buffer containing the heavy metal quencher and eserine. In addition to thereby establishing an agonistic action of eserine at *Torpedo* nAChR, our studies produced several novel findings. The agonistic action of eserine could not be blocked by established cholinergic antagonists, including D-tubocurarine, α-bungarotoxin and the competitive antibody WF6 [22]. On the contrary, under these conditions of blocked acetylcholine binding sites, eserine uninhibitedly activated the nAChR channel. Searching for compounds that antagonize the channel-activating action of eserine, we established two monoclonal antibodies and a low-molecular-mass compound that competed with eserine in binding to *Torpedo* membrane fragments. Taken together, eserine exerts its agonistic effects from sites at the nAChR independent of those for the natural transmitter, and thus may mimick properties of an (as yet unidentified) endogenous ligand of the nAChR.

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Abbreviation. nAChR, nicotinic acetylcholine receptor.

EXPERIMENTAL PROCEDURES

Preparation of membrane vesicles

mAChR-rich membrane vesicles were prepared from *Torpedo marmorata* electric tissue according to [23] with the minor modifications described in [19]. Following sucrose gradient fractionation, the fraction with the highest concentration of nAChR was pooled, diluted with a 10-fold excess of ice-cold distilled water and centrifuged for 30 min at 18000 rpm in an SS 34 rotor. The pellet was resuspended in 300 mM NaCl, 10 mM Hepes pH 7.0 and stored at −80 °C. The receptor concentration of the suspension was generally 17 − 20 μM in terms of acetylcholine binding sites at a protein concentration of 10 − 14 mg/ml.

Ion flux studies

Ion flux studies were performed according to [16] with the following modifications: 1,3,6,8-pyrene tetrasulfonate was used as fluorescent dye, and Cs^+ instead of Tl^+ was used as heavy metal quencher [21]. Loading of the membrane vesicles with dye was achieved by three cycles of freezing (in liquid nitrogen) and thawing (in ice water). Excess dye was removed by passage through a column of Sephadex G-25 (coarse) equilibrated with Na/Hepes buffer (300 mM NaCl, 10 mM Hepes pH 7.0), the elution time being approximately 15 min. The eluate was then made 50 μM in the esterase blocker Tetram. The receptor concentration of the vesicle suspension after passage through the column was of the order of 1 μM acetylcholine binding sites.

The vesicle suspension was rapidly mixed in a HighTech SF-51MX stopped-flow fluorimeter with an equal volume of Cs/Hepes buffer (300 mM CsCl, 10 mM Hepes pH 7.0) with or without activating ligand. Excitation of fluorescence was by a 240-W xenon/halogen lamp with the light being passed through a Schott UG 11 ultraviolet broad-band filter combined with a UG 11 RB filter. The emitted light was passed through a Schott KV 399 filter before reaching the photomultiplier. After A/D conversion, the signals were collected in a personal computer equipped with a Sorcus modular IV card. The reported kinetic traces were each averaged from at least five independent experiments.

Ion flux experiments were performed at 19.6 °C within 90 min after loading of the vesicles. Preincubation with small ligands (D-tubocurarine, dibucaine, acetylcholine) was performed with dye-loaded vesicles for 30 min; preincubation with high-molecular-mass ligands (neurotoxins, antibodies) was performed overnight prior to loading the vesicles with fluorescent dye. All experiments were performed in the presence of the esterase blocker Tetram. Tetram itself did not show any activity towards the nAChR.

Binding experiments

Binding of tritiated acetylcholine or eserine to membrane-bound nAChR was performed with crude *Torpedo* membrane preparations [24]. The stock suspension of membranes was diluted into 100 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Pipes pH 6.8, supplemented with 0.5 mg/ml bovine serum albumin, repeatedly shock frozen in liquid nitrogen (to open endogenous presynaptic vesicles), and finally supplemented with 0.125 mM Tetram. Aliquots of 400 μl were transfered into Eppendorf reaction vials and, after addition of the appropriate ligands, the total reaction volume was adjusted to 500 μl with buffer. After incubation for 30 min at room temperature (in the presence of eserine in the dark), the vials were centrifuged under refrigeration (4 °C) in an SS34 rotor at 20000 rpm for 60 min. The free concentration of radioactive ligand was determined from 100-μl aliquots of the supernatant. After careful removal of the supernatant by suction and absorption by the tip of a paper towel, the bottom of the reaction vial was clipped off, and the radioactivity in the pellet (bound ligand) was determined. Nonspecific binding increased linearily with the concentration of radioactive ligand employed. It was determined by linear regression and substracted from total radioactivity bound.

Binding of antibody FK1 to membrane-bound nAChR was studied by ELISA [25, 26] employing either crude or highly purified membrane preparations. For quantitative analysis, the monoclonal antibody was purified to apparent homogeneity [22], the total concentration of immobilized nAChR was determined in control wells by binding of α-[^{3}H]bungarotoxin, the enzyme reactions were performed under strictly controlled conditions, and all absorbance determinations were performed within the linear range of the spectrophotometer (Kuhlmann, J. and Maelicke, A., unpublished results). Assuming 1 : 1 stoichiometry between antibody and nAChR [22], the saturation level of absorbance is then correlated with the concentration of immobilized nAChR per well, and the ELISA curves can be analysed in terms of binding equilibria. With the concentration of antibody and its affinity of binding to the nAChR known, competition of eserine and antibody for receptor binding can be studied. From the concentration of half-maximal competition, an apparent K_i value can then be calculated.

RESULTS

Eserine can activate the Torpedo *nAChR channel even in the presence of cholinergic antagonists*

Fig. 1 summarizes the results of Cs^+ influx studies employing fluorescent dye-loaded nAChR-rich *Torpedo* membrane vesicles and eserine. As shown in Fig. 1A, eserine-induced quenching of fluorescence exhibited similar properties as previously observed by the same assay for acetylcholine and its agonists [16 − 18, 22]. By inference, the initial rapid decrease in fluorescence is induced by the opening of eserine-gated nAChR channels, with the slow phase of approach to leakage flux levels representing desensitization of the same channels [15 − 18]. As a control, the dye-loaded vesicles were disrupted by the addition of organic solvent, e.g. 2% dimethylsulfoxide, so that the total amount of vesicle-enclosed dye was exposed to the heavy metal quencher. The amplitude of fluorescence quenching resulting from this treatment was several times larger than the maximal amplitude of eserine-induced quenching, showing that the latter was not limited by exhaustion of free dye. The concentration dependence of fluorescence quenching could therefore be used to construct a dose/response relationship resulting in an eserine concentration required for half-maximal response of 300 − 500 μM. This concentration is approximately one order of magnitude higher than that determined for acetylcholine [1, 3, 18].

The other panels of Fig. 1 demonstrate that eserine can activate the *Torpedo* nAChR channel even in the presence of antagonists of acetylcholine but not in the presence of the noncompetitive inhibitor dibucaine. Fig. 1B contains the traces of six different experiments, the first four of which representing only leakage influx of Cs^+ into the vesicles. The latter was observed in the absence of any activating ligand

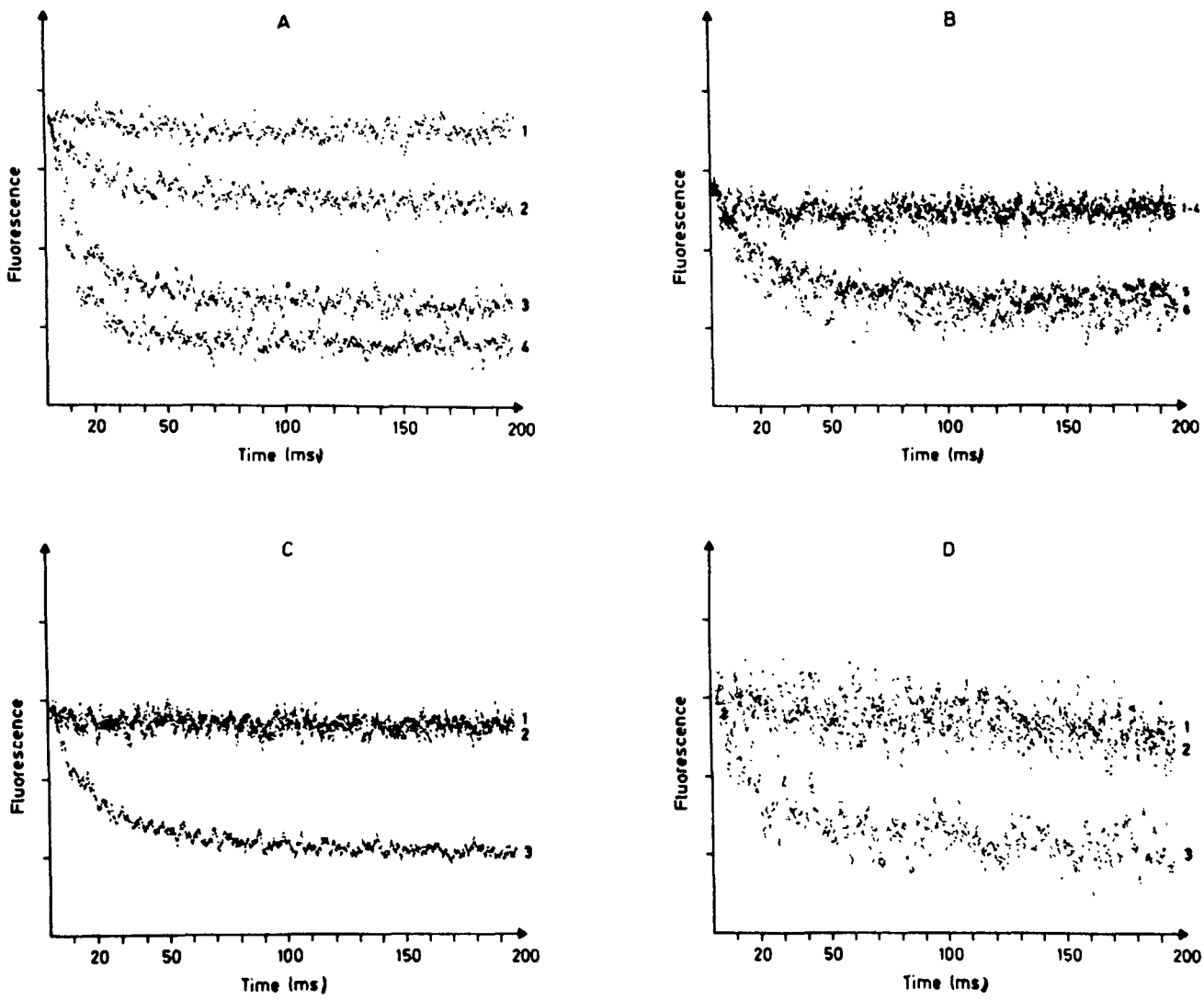

Fig. 1. *Eserine-stimulated Cs^+ influx into membrane vesicles from* T. marmorata *electric organ in the absence and presence of antagonists of acetylcholine.* (A) Concentration dependence of Cs^+ influx stimulated by eserine. Membrane vesicles (concentration of acetylcholine binding sites approx. 1 μM), loaded with 1,3,6,8-pyrene tetrasulfonate and suspended in Na/Hepes buffer were rapidly mixed with Cs/Hepes buffer in the absence (trace 1) and presence (traces 2, 3, 4) of (−)-physostigmine (D-eserine). The change in fluorescence was recorded as a function of time. Trace 1 represents uninduced 'leakage' flux, i.e. the spontaneous equilibration of Cs^+ between extravesicular and intravesicular space in the absence of channel-activating ligands. Traces 2, 3 and 4 were obtained when Cs/Hepes buffer was supplemented with eserine to a final concentration (after mixing) of 115 μM, 920 μM and 1.84 mM, respectively. Each trace represents the average of five experiments. From a secondary plot of the quenching amplitudes versus the concentration of eserine applied, a half-maximal concentration for channel activation of 350−400 μM was obtained. (B) D-Tubocurarine and decamethonium do not antagonize eserine-stimulated Cs^+ influx. Under similar experimental conditions as described above, dye-loaded vesicles were incubated for 15 min with 200 μM decamethonium (trace 2) or tubocurarine (trace 3) or benzoquinonium (trace 4) followed by rapid mixing with an equal volume of Cs/Hepes buffer supplemented with 20 μM acetylcholine. The observed fluorescence decay curve was indistinguishable from leakage flux (trace 1) suggesting that the concentration of inhibitor fully sufficed to block all cholinergic sites available. In contrast, when dye-loaded vesicles, preincubated with 200 μM decamethonium, were rapidly mixed with Cs/Hepes buffer supplemented with 200 μM eserine rather than acetylcholine, almost the same level of channel activation (trace 5) as in the absence of decamethonium (trace 6) was observed. A similar kinetic as trace 5 was observed when the vesicles were preincubated with D-tubocurarine. (C) Antibody WF6 does not antagonize eserine-stimulated Cs^+ influx. Traces 1 and 2, membrane vesicles were pretreated overnight at 4 C with antibody WF6 (≈ 50-fold excess over acetylcholine binding sites), loaded with 1,3,6,8-pyrene tetrasulfonate and then rapidly mixed with an equal volume of 40 μM acetylcholine in Cs/Hepes buffer. Only leakage flux was observed. Trace 3, same experimental conditions as above expect that acetylcholine was replaced by 400 μM eserine. (D) The local anaesthetic dibucaine inhibits eserine-stimulated Cs^+ influx. Trace 1, dye-loaded vesicles were rapidly mixed with Cs/Hepes buffer in the absence of activating ligands (leakage flux). Trace 3, dye-loaded vesicles were rapidly mixed with Cs/Hepes buffer containing 200 μM eserine. Trace 2, dye-loaded vesicles were preincubated for 30 min with 200 μM dibucaine followed by rapid mixing with Cs/Hepes buffer containing 200 μM eserine

(trace 1), and also in the presence of 20 μM acetylcholine when the vesicles were preincubated (prior to rapid mixing with acetylcholine in Cs/Hepes buffer) with either 200 μM decamethonium (trace 2) or 200 μM D-tubocurarine (trace 3) or 200 μM benzoquinonium (trace 4). Trace 5 was observed when dye-loaded vesicles pretreated with 200 μM decamethonium (or 200 μM D-tubocurarine) were rapidly mixed with Cs/Hepes buffer containing 200 μM eserine instead of acetyl-

choline. Clearly, the employed concentrations of decamethonium and D-tubocurarine sufficed to inhibit activation of the receptor channel by acetylcholine while activation by eserine uninhibitedly proceeded to a similar, if not identical, level as in the absence of these cholinergic antagonists (trace 6). Hence, since the affinity of eserine binding to the receptor is much below that of acetylcholine (see below), eserine appears to activate the observed ion flux from other than the acetylcholine sites.

Similarly (Fig. 1C), preincubation of dye-loaded vesicles with the acetylcholine-competitive antibody WF6 [22, 25] blocked channel activation by acetylcholine but not by eserine. The same was observed for antibody WF5 and the neurotoxin α-bungarotoxin (not shown here). In contrast, the local anaesthetic dibucaine, an established open channel blocker of acetylcholine activation [1, 3], also blocked channel activation by eserine (Fig. 1D) suggesting that acetylcholine and eserine indeed act on the same channel, albeit from independent binding sites at the receptor.

The carbamate structure is not required for nAChR channel activation by eserine

Physostigmine, at neutral pH, is a positively charged tricyclic amino compound with a methylcarbamate side chain. Binding to acetylcholine esterase is assumed to occur via the enzyme's 'anionic site' which places the carbamate group in apposition to the 'esteratic site' of acetylcholine esterase [4].

To test whether carbamoylation of the receptor is associated with the channel-activating property of eserine, we hydrolysed away the *N*-methylcarbamate group (the truncated eserin acts as reversible competitive inhibitor rather than as nearly irreversible inhibitor of acetylcholine esterase) and performed ion flux studies as described above. Truncated eserine exhibited the same channel-activating potency as eserine, suggesting that the carbamate function is neither required for binding to the receptor, nor for channel activation. This agrees with the inability of previous studies to identify active serine residue(s) at the nAChR (by means of active-site titration with [^{3}H]diisopropylfluorophosphate, [27, 28]).

For the same reason, the phosphorothiolate Tetram does not activate or interfere with nAChR channel activation by cholinergic agonists or by eserine. Tetram was hence used as an anticholinesterase in most of our experiments (a) to exclude enzymatic hydrolysis of acetylcholine esterase substrates, and (b) to reduce, if possible, the interaction of eserine with the remaining traces of acetylcholine esterase in our preparation.

Antibody FK1 and benzoquinonium antagonize channel activation by eserine

We screened an available library of monoclonal antibodies raised against nAChRs from different species for members inhibiting eserine-induced ion flux into dye-loaded vesciles. None of our antibodies raised against *Torpedo* nAChR [22, 25, 29] displayed such properties, but two others were found that did. Antibody FK1 (Fels, G., Kuhlmann, J. and Maelicke, A., unpublished) was raised against hindleg muscle cell membranes from newborn rats [30]; antibody PK1 was raised against a synthetic peptide conjugated to bovine serum albumin [31]. As shown for FK1 in Fig. 2A, preincubation with saturating concentrations of the antibody completely inhibited Cs^+ influx into *Torpedo* membrane vesicles, establishing the antibody as a high-molecular-mass antagonist of

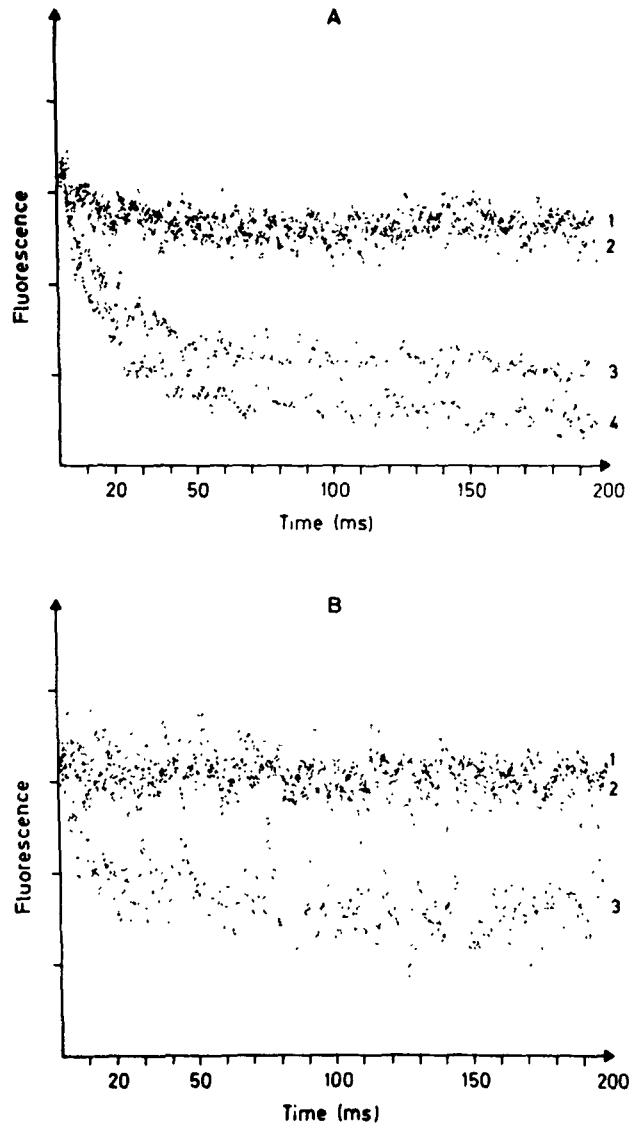

Fig. 2. *Eserine-stimulated Cs^+ influx into* T. marmorata *membrane vesicles in the presence and absence of antibody FK1.* (A) Antibody FK1 antagonizes eserine-stimulated Cs^+ influx. Traces 1 and 2, membrane vesicles were pretreated overnight at 4°C with antibody FK1, loaded with dye and rapidly mixed with equal volumes of 200 μM eserine or 400 μM eserine in Cs/Hepes buffer. Both experiments yielded similar traces suggesting that the concentration of FK1 employed was close to saturation. (The remaining small component of rapid fluorescence quenching may be due to insufficient time of preincubation and/or relatively low affinity of binding of antibody FK1.) Traces 3 and 4, same experimental conditions as above except that the membrane vesicles were not pretreated with antibody FK1. (B) Benzoquinonium antagonizes eserine-stimulated Cs^+ influx. Dye-loaded membrane vesicles were pre-treated for 15 min with 100 μM benzoquinonium followed by rapid mixing with an equal volume of Cs/Hepes buffer supplemented with (trace 2) or without (trace 1) 200 μM eserine. Trace 3, dye-loaded membrane vesicles were not treated with benzoquinonium prior to rapid mixing with 200 μM eserine in Cs/Hepes buffer

eserine. This finding is further supported by the binding studies reported below.

From an extensive screening program to identify low-molecular-mass antagonists of eserine, we found that the cholinergic antagonist benzoquinonium [32] inhibits the ion-flux-inducing activity of both acetylcholine and eserine (Fig. 2B).

This result may be of particular interest with respect to the location of the eserine site(s) at the receptor. As no evidence for other than acetylcholine-competitive benzoquinonium binding sites at the nAChR exists [32], it is likely therefore that eserine and acetylcholine bind to neighboring sites at the receptor's α-subunit (with the binding site of benzoquinonium overlapping with both sites).

Binding of eserine to Torpedo *membrane fragments*

Direct binding of [^{3}H]eserine to membrane-bound nAChR is difficult to assess because, even under equilibrium conditions, the binding affinity remains low. The high concentrations of membrane-bound receptor therefore required for binding studies [28], even if available, obscures the proper separation of solute and particulate phase required by the usual binding assays [24, 33, 34]. Therefore forced to working at receptor concentrations below 1 μM (Fig. 3A), we estimated for eserine a K_d value of the order of 50–100 μM. Thus, persistent occupation of nAChR by eserine increases the affinity of eserine binding by only one order of magnitude, while desensitization of the receptor by acetylcholine is accompanied by an increase in binding affinity for acetylcholine of approximately three orders of magnitude [24, 34, 35].

Fig. 3B displays the results of competition binding studies with eserine and [^{3}H]acetylcholine. Only very weak inhibition of [^{3}H]acetylcholine binding was observed, resulting in a K_i value for eserine in the millimolar range. Obviously, the acetylcholine-competitive eserine binding site(s) differ in affinity and probably also in location from those responsible for the eserine-induced ion flux. They rather appear to represent the sites of noncompetitive antagonism (direct channel blockade) previously identified by electrophysiological experiments [8–13].

As a further approach to determine the affinity of binding of eserine to the nAChR, its competition with antibody FK1 for receptor binding was studied (Schrattenholz, A. and Maelicke, A., unpublished). Calculations based on the concentration of eserine required for 50% reduction of FK1 binding to *Torpedo* nAChR yielded K_i values of the order of 20 μM, which is in reasonable agreement with the results of direct binding studies (Fig. 3A).

The existence of a high-affinity antagonist of eserine (antibody FK1) permits assessment of the pharmacology of eserine-induced ion flux on the level of the binding reaction [28]. In agreement with the findings reported above, only eserine and benzoquinonium but not acetylcholine, tubocurarine or decamethonium inhibited, at the concentrations applied, binding of FK1 to *Torpedo* membrane fragments.

Taken together, our binding studies yielded the following findings. (a) The existence of eserine-competitive antibodies suggests that the channel-activating binding site(s) for eserine is located at the surface, not inside the channel of the nAChR. (b) The eserine binding site is probably adjacent to the acetylcholine site because benzoquinonium competes in binding with both ligands (thereby inhibiting channel activation). (c) Blockade of the acetylcholine-activated channel [8–13] is probably exerted from other site(s) than direct channel activation by eserine because of the very different affinity range of these sites.

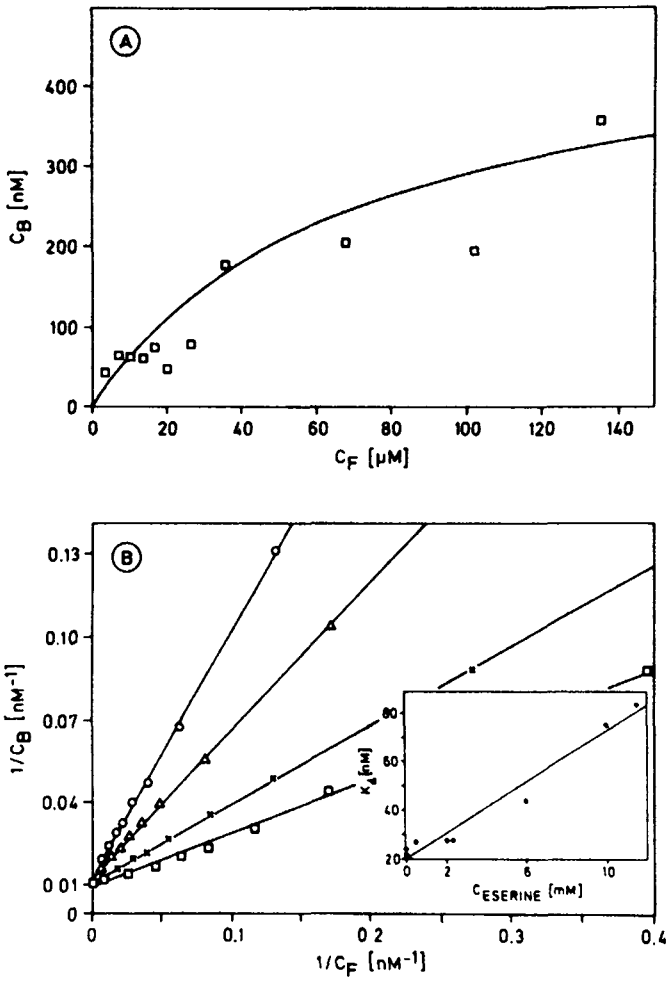

Fig. 3. *Binding of eserine to membrane-bound nAChR from* T. marmorata. (A) Binding of [^{3}H]eserine to *Torpedo* membrane fragments. *Torpedo* membrane fragments (approx. 500 nM acetylcholine binding sites) in 100 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Pipes pH 6.8, supplemented with 0.4 mg/ml bovine serum albumin and 0.1 mM Tetram, were incubated, in the dark and at room temperature, for 30 min with different concentrations of [^{3}H]eserine (2–170 μM). After centrifugation of the reaction mixture, the concentration of free eserine was determined from the radioactivity of an aliquot of the supernatant, the concentration of bound eserine from the radioactivity of the pellet. Nonspecific binding was determined in the presence of 20 mM nonradioactive eserine. The data are plotted in terms of the concentration of bound (c_b) versus free (c_f) eserine. They were fitted by assuming a single class of binding sites, a concentration of sites of 500 nM, and a K_d value of 70 μM. (B) Binding of [^{3}H]acetylcholine to membrane-bound nAChR in the presence and absence of fixed concentrations of eserine. Under the same experimental conditions as described above, *Torpedo* membrane fragments (approx. 100 nM acetylcholine binding sites) were incubated at room temperature for 30 min with different concentrations of [^{3}H]acetylcholine (0.02–1 μM) in the presence of no (○), 2 mM (△), 6 mM (×) and 10 mM (□) eserine, respectively. After separation by centrifugation of solute and particulate phase, the radioactivity in the supernatant and in the pellet were determined. Data are presented in a double-reciprocal plot of the concentration of bound acetylcholine (c_b) versus free (c_f) acetylcholine. From a secondary plot of the apparent K_d values for acetylcholine binding versus the concentration of eserine (additional experiments included), a K_i value of 3.9 mM was obtained (linear regression, correlation coefficient $r^2 = 0.996$)

DISCUSSION

Our data extend previous electrophysiological and biochemical observations. The ion flux studies employing pre-nAChR-rich *Torpedo marmorata* membrane vesicles (Figs 1 and 2) are in qualitative and quantitative agreement with

vious electrophysiological studies on nAChR from other species [8–13] suggesting that our findings may not be limited to only *Torpedo* receptor. As the major novel observation, eserine is capable of activating the *Torpedo* nAChR channel, and this activation is not antagonized by most antagonists of acetylcholine (including D-tubocurarine and α-bungarotoxin) but is antagonized by the monoclonal antibody FK1 and by benzoquinonium. Further supported by our binding studies, we established in this way a new class of channel-activating site(s) at the *Torpedo* nAChR that is clearly distinct in location and pharmacology from the cholinergic sites. Epitope mapping studies employing synthetic peptides [25, 36] and affinity labeling studies are presently under way in order to locate conclusively in the primary structure of the nAChR the site from which eserine exerts its ion-flux-stimulating activity. In addition to these sites, separate sites exist (probably the local anaesthetic binding sites previously identified [37, 38]) from which eserine, at high concentrations, acts as a direct blocker of the acetylcholine-activated channel [12].

Working with the nAChR from the mollusc *Aplysia*, Carpenter et al. [39] have observed that eserine blocks binding of α-bungarotoxin with an inhibition constant of 3 μM. By intracellular recording from *Aplysia* neurons, the same authors reported that eserine (100 μM) 'inhibits' the block by α-bungarotoxin of acetylcholine channel activation. The latter finding may have been caused by the direct channel activating effect of eserine described here, while the first suggests that, at the *Aplysia* nAChR, the binding sites for α-bungarotoxin and eserine may partially overlap.

At the nAChR from *Torpedo nobiliana* electric organ, high-affinity binding sites for eserine ($K_d \approx 20-50$ μM) have been found which apparently overlap not only with those for α-bungarotoxin but also with those for acetylcholine. In this system eserine and α-bungarotoxin compete for nAChR binding, and eserine acts as a cholinergic antagonist [40].

At the neuromuscular junction of mammals, α-bungarotoxin and other antagonists of acetylcholine are known to block neuromuscular transmission completely. Variations in the pharmacology of nicotinic receptors from different species, organs and brain regions are well documented [2, 41]. In view of the inherent limits of our *in vitro* assay, the possibility also exists that eserine and acetylcholine bind to the same general binding pocket at the *Torpedo* nAChR but that binding occurs via different attachment points [25, 26].

Focussing again on the key findings of this study, (−)-physostigmine, in the true sense, is not an agonist of acetylcholine at *T. marmorata* nAChR but rather represents a new class of its own of channel-activating ligands of the receptor. Accordingly, this class of sites has its own agonists (eserine) and antagonists (FK1, benzoquinonium). This raises the question as to whether the channel-activating binding sites for eserine serve a physiological function controlled by a natural (endogenous) ligand. In other words, the nAChR, in addition to serving as receptor for acetylcholine, might be the receptor for yet another messenger molecule. Several compounds known to affect nAChR function and development may be considered as candidate messengers in this context [42–45]. A similar multiplicity of receptive functions is not unknown for neuroreceptors serving as ligand-gated cation channels [46–49].

The initial part of this study was performed at the Max-Planck-Institut für Ernährungsphysiologie, Dortmund, FRG. Some of the later studies (in Mainz) were performed with the participation of Dr A. Schrattenholz, T. Coban and B. Schröder. This work was supported by the *Deutsche Forschungsgemeinschaft*, the *Alexander-von-Humboldt foundation* (by a fellowship for K.O.), and the *Fonds der Chemischen Industrie*. We thank E.X. Albuquerque and B.M. Conti-Tronconi for fruitful discussions. The excellent technical assistance of G. Wehmeyer is gratefully acknowledged.

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