Eserine and Other Tertiary Amine Interactions with *Torpedo* Acetylcholine Receptor Postsynaptic Membrane Vesicles†

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ABSTRACT: Interaction of the tertiary amines, arecolone, eserine (physostigmine), (+)-epibatidine, and (±)-epibatidine, with *Torpedo* nicotinic acetylcholine receptor-enriched membrane vesicles was investigated to characterize their action on the receptor, using stopped-flow thallium (I)-flux spectrofluorimetry. Arecolone, (+)-epibatidine, and (±)-epibatidine were agonists with activation constants of 390, 19, and 39 μM, respectively. Eserine was not an agonist but rather an antagonist for agonist-induced activation of the receptor with an inhibition constant of ~150 μM. The choice of the fluorescent dye used (entrapped within the membrane vesicles) was critical for interpretation of the effects of eserine. With 1,3,6,8-pyrene tetrasulfate (PTS), eserine appeared to act as an agonist. However, it was shown that such an effect was caused by rapid diffusion of the uncharged form of the amine across the membrane followed by direct interaction with PTS rather than eserine-induced cation transport. The use of a different fluorescent dye, 8-aminonaphthaline-1,3,6-trisulfate, with which eserine does not interact allowed demonstration of the action of eserine as an antagonist rather than as an agonist.

The *Torpedo* nicotinic acetylcholine receptor (nAcChR)[1] is a ligand-gated ion channel composed of four subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ (reviewed in *1*) and resembles most closely the muscle-type nAcChR. Two agonists bind to activation sites and open a channel and induce cation flux, which results in depolarization of postsynaptic cell membranes. Abundance of this receptor in the electric tissue has allowed extensive biochemical studies and this nAcChR serves as a model for other ligand-gated ion channels, which include neuronal nAcChR, γ-aminobutyric acid type A ($GABA_A$), glycine, and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors (*1, 2*).

The study of structure−activity relationships between cholinergic agonists and their binding site(s) on nAcChRs has led to identification of very potent compounds with a semirigid structure such as the quaternary amines isoarecolone-methiodide and arecolone-methiodide (*3, 4*). Similarly, anatoxin-a, a secondary amine with a bicyclic structure (*5*−*7*) and epibatidine, a tertiary amine also containing a secondary amine with a bicyclic structure (*8*−*11*) are also very potent. In addition activation of the *Torpedo* receptor was reported to be induced by the tertiary amine eserine (physostigmine), an AcChE inhibitor, through a mechanism distinct from that for conventional agonists such as AcCh or Carb (*12*). Eserine, a carbamate derivative of a tertiary amine, readily crosses the blood-brain barrier and is used centrally as well as peripherally for reversible inhibition of AcChE. Previous electrophysiological studies reported that eserine had depolarizing effects through direct interaction with the nAcChR on cultured skeletal muscle cells (*13*). Activation of nAcChR single channels by this AcChE inhibitor was also reported, although contradictory results were obtained depending on the preparation used (reviewed in *14*). Patch-clamp studies revealed that eserine does open nAcChR single channels of frog skeletal muscle fibers (*15*).

Stopped-flow, ion-flux methods with fluorophore-loaded, nAcChR-enriched membrane vesicles from *Torpedo* electric tissue have been useful for characterizing nAcChR activation, because kinetic parameters and activation dissociation constants can be obtained and compared with values obtained from electrophysiological studies (*16*−*18*). Using this technique, we investigated the activation properties of tertiary amines such as arecolone, a precursor of the potent nicotinic agonist arecolone-methiodide, eserine (physostigmine), an AcChE inhibitor with a tertiary amine functionality, and the enantiomeric mixture (±) and dextrorotatory forms (+) of epibatidine, a potent nicotinic agonist (*8, 11*). Arecolone, (±)-epibatidine, and (+)-epibatidine were found to act as agonists for *Torpedo* nAcChR. Eserine, however, did not activate the nAcChR of *Torpedo* membrane vesicles. Rather, eserine inhibited the cation flux induced by Carb and other agonists in a concentration-dependent manner with IC_{50}

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[1] Abbreviations nAcChR, nicotinic acetylcholine receptor; AcCh, acetylcholine; Carb, carbamylcholine; arecolone, arecolone-HCl; AcChE, acetylcholinesterase; PTS, 1,3,6,8-pyrene tetrasulfate; ANTS, 8-aminonaphthaline-1,3,6-trisulfate disodium salt; α-BTx, α-bungarotoxin; *d*-Tc, *d*-tubocurarine; MTPP, methyltriphenylphosphonium-bromide; BSA, bovine serum albumin; NTE, triethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazole; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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values that were similar for all agonists tested. The previously observed apparent activation of *Torpedo* receptor by this AcChE inhibitor (*19, 20*) can be explained by the fact that it readily traverses the lipid bilayer of the vesicles, interacts directly with and quenches the fluorescence indicator PTS loaded inside the vesicles, thereby producing apparent activation responses in such stopped-flow experiments.

EXPERIMENTAL PROCEDURES

Materials. Carbamylcholine chloride, eserine, and bovine serum albumin (BSA) were from Sigma; 8-amino-1,3,6-naphthalene trisulfonic acid disodium salt (ANTS) and 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt (PTS) were from Molecular Probes Inc.; methyltriphenylphosphonium bromide (MTPP) was from Pfaltz and Bauer, Inc. Arecolone-HCl was provided by Dr. John S. Ward (Lilly Reserach Laboratories, Indianapolis, IN) and Dr. C. E. Spivak (National Institutes of Health, Baltimore, MD). (±)-Epibatidine was from Tocris Cookson (Bristol, UK). (+)-Epibatidine was from RBI (Natick, MA). The electric organs of *Torpedo californica* were from Aquatic Research Consultants (San Pedro, CA).

Membrane Preparation, Treatment, and Fluorophore Loading. The nAcChR-enriched membrane vesicles were prepared from *T. californica* as described previously (*21*) and were alkali-treated to remove peripheral proteins (*22, 23*). Unless otherwise indicated, triethanolamine (NTE) was used as a buffer at pH 7.4. After centrifugation at 18 000 rpm and 4 °C for 45 min with Sorvall RC5C and SS-34 rotors, the membranes were resuspended and incubated in buffer containing BSA (20 mg/mL BSA, 10 mM NTE, pH 7.4, 0.02% NaN_3) for 90 min twice and then overnight. BSA was removed by resuspending and diluting the membranes in 40 mL NTE buffer (10 mM NTE, pH 7.4, 0.02% NaN_3) followed by centrifugation as described above. After centrifugation and resuspension, one part of the membrane preparation was mixed with two parts of 25 mM ANTS or 21 mM PTS prepared in NTE buffer at a final pH of 7.4. To load the fluorophore into the vesicles, the fluorophore-membrane preparation was frozen in liquid nitrogen and thawed in an ice-cold bath. The freeze−thaw cycle was then repeated. External fluorophore was removed using a Sephadex G-25−300 column (1.0 × 20 cm) equilibrated in Na^+-buffer (35 mM $NaNO_3$, 10 mM NTE, pH 7.4, 0.02% NaN_3).

Membrane Lipid Extraction and Preparation of Dye-Loaded Lipid Vesicles. The extraction of lipids from *T. californica* electric organs was performed essentially as described by Bligh and Dyer (*24*), except that the water content of the tissue was assumed to be 90%. Larger solid material was removed by filtration through cheesecloth, and the filtrate was filtered through Whatman No. 1 filter paper with slight suction. The final filtrate was transferred to a separatory funnel and after separation the bottom chloroform portion was collected in a round-bottom flask and the chloroform evaporated using a rotary evaporator. The lipid sample was stored in vacuo.

Fifty milligrams of the lipid was mixed with 5 mL NTE buffer and stirred for 90 min at room temperature. The mixture was sonicated for 1 h under argon, resulting in a white suspension. No protein, including nAcChR subunits, was observed in SDS−PAGE. PTS was vortex-mixed with the lipid solution to obtain a PTS concentration of 17 mM. The PTS-lipid vesicle solution was subjected to a freeze−thaw cycle twice. The PTS-loaded lipid vesicles were collected from a Sephadex G-25−300 column to remove extravesicular PTS and then used in stopped-flow experiments.

Thallium(I) Flux Stopped-Flow Experiments and Analysis. The stopped-flow measurements of Tl^+ flux through vesicles were described previously (*16*). Fast kinetic measurements of fluorescence changes caused by quenching of fluorescence by Tl^+ within the vesicles were obtained by use of an SF.17MV MicroVolume Stopped-flow Spectrofluorimeter apparatus (Applied Photophysics Ltd., Leatherhead, UK) with a xenon arc lamp (150 W) for excitation at both 370 nm for ANTS and 382 nm for PTS. Appropriate filters for fluorescence emission were used for each fluorophore. A suspension of fluorophore-loaded vesicles was rapidly mixed with an equal volume of Flux buffer (35 mM $TlNO_3$, 10 mM NTE, pH 7.4, 0.02% NaN_3), containing a ligand or a mixture of ligands at 25.0 ± 0.2 °C. For experiments without Tl^+, the Na^+-buffer replaced the Flux buffer. Control (leak) traces were obtained in the absence of ligand in the Flux buffer. Fluorescence quenching data were acquired and analyzed by a connected Acorn computer with kinetic software provided by Applied Photophysics Ltd. Each kinetic trace was fit to the following modified Stern−Volmer equation (*16*):

$$F(t) = A_1/[1 + KT_\infty(1 - e^{-kt})] + k_0 t + A_0$$

where $F(t)$ is the fluorescence intensity at time t, A_1 is the fluorescence intensity in the absence of Tl^+, K is the Stern−Volmer constant for a fluorophore (ANTS or PTS), T_∞ is the maximum concentration of Tl^+ inside the vesicles, k is the apparent rate constant of Tl^+ flux, k_0 is the rate of Tl^+ leak through the membrane, and A_0 is the fluorescence intensity for the baseline. The log concentration-rate curves were plotted and fit to the following sigmoidal curve equation using InPlot4 software (GraphPad Software Inc.):

$$Y = A + (B - A)/[1 + (10^X/10^C)^D]$$

where Y is the apparent rate (k_{app}), A is the minimum rate, B is the maximum rate (k_{max}), X is the log of ligand concentration, C is log K_{act}, and D is the slope constant or "pseudo" Hill coefficient.

Recording of Fluorescence Emission Spectra. Fluorescence emission spectra of PTS and ANTS were obtained using a Perkin-Elmer MPF-44A fluorescence spectrophotometer. In a 10-mm quartz cuvette, 25 mM ANTS or PTS was mixed with an appropriate volume of HEPES buffer (10 mM HEPES, pH 7.4, 0.02% NaN_3) in the absence or presence of a given amine, and emission spectra were recorded with excitation wavelength of 370 nm for ANTS or 382 nm for PTS. At these wavelengths, emission spectra for HEPES buffer or for the amines alone in the buffer were negligible.

For obtaining the spectra of PTS at various pH values, 25 mM PTS and 2 mM eserine were prepared in Tris buffers (10 mM Tris-Cl) for pH 7 to 9 and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffers (10 mM Na^+-MES) for pH 5 and 6. Equal volumes of 25 mM PTS and buffer or 2 mM eserine of identical pH values were mixed for the spectra of

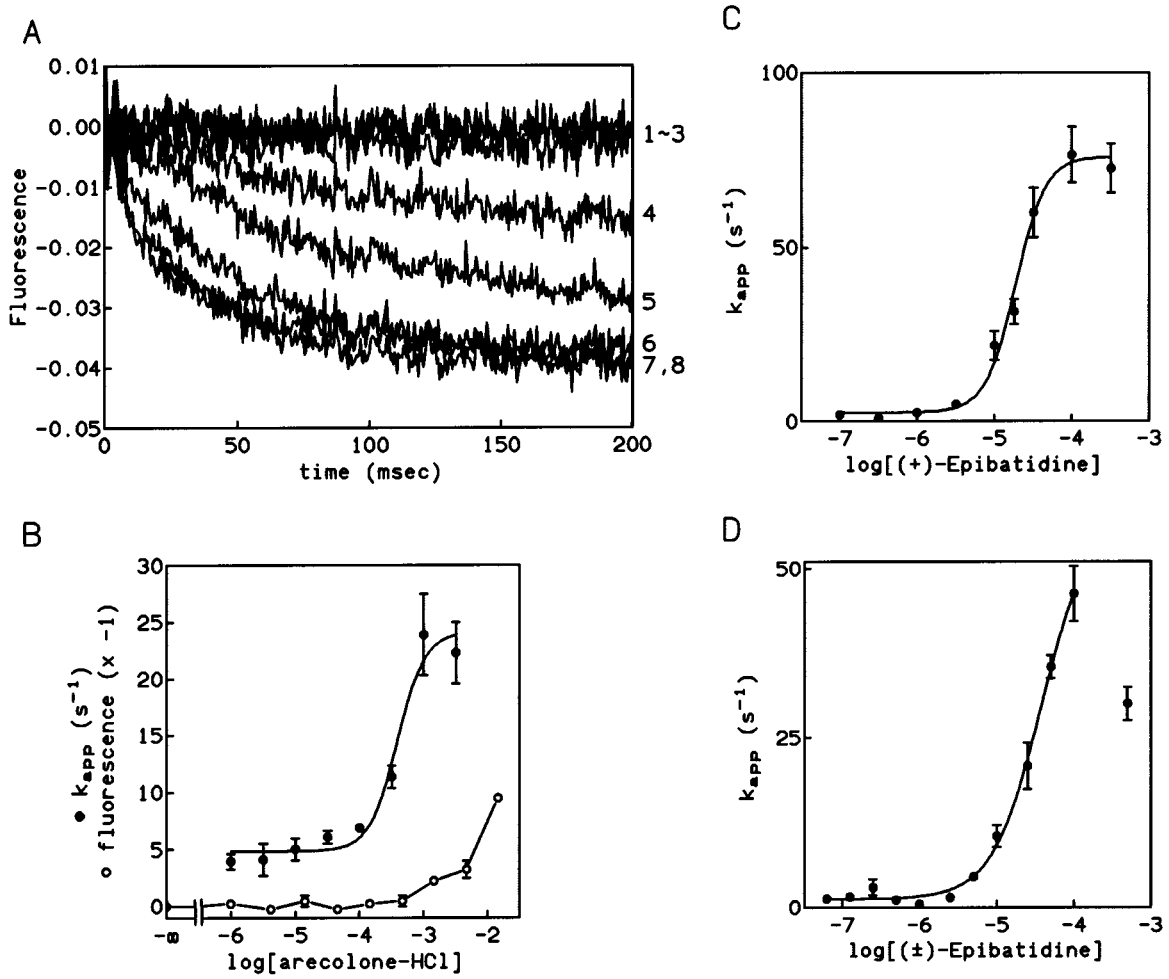

FIGURE 1: Stopped-flow Tl^+-flux responses for arecolone, (+)-epibatidine, and (±)-epibatidine. ANTS-loaded *Torpedo* membrane vesicles were subjected to Tl^+-flux experiments. (A) Kinetic fluorescence traces for arecolone. Concentrations used were 0 μM (trace 1), 3.2 μM (trace 2), 10 μM (trace 3), 32 μM (trace 4), 100 μM (trace 5), 320 μM (trace 6), 1 mM (trace 7), and 3.2 mM (trace 8). Each trace was obtained by averaging individual traces for each concentration and by subtracting averaged control (leak) traces. (B) (●) The apparent rate for each concentration was obtained by averaging rates of at least 4 traces (mean ± SEM) and was plotted as a log concentration of arecolone. K_{act} and k_{max} were 390 μM and 24 s^{-1}, respectively. The rate for 320 μM Carb was 150 ± 30 s^{-1}. (○) Relative fluorescence of ANTS titrated with arecolone. Emission fluorescence of ANTS was measured as described in Materials and Methods and is indicated as a function of cumulative arecolone concentrations. Each point is mean ± SEM from duplicate measurements. (C) Concentration−response curve for (+)-epibatidine. Sigmoid curve fitting resulted in $k_{max} = 76$ s^{-1}, log $K_{act} = -4.72$ ($K_{act} = 19$ μM), and $n = 2.1$. The rate for 320 μM Carb for this preparation was 100 ± 6 s^{-1}. HEPES was used instead of NTE for buffer. (D) Concentration−response curve for (±)-epibatidine. $k_{max} = 59$ s^{-1}, log $K_{act} = -4.41$ ($K_{act} = 39$ μM), and $n = 1.4$. The rate for 500 μM Carb for this preparation was 30 ± 3 s^{-1}.

12.5 mM PTS alone or 12.5 mM PTS and 1 mM eserine. Emission spectra for buffers with or without the amines were negligible.

For the emission fluorescence titration of ANTS with arecolone, 2 mL of 12.5 mM ANTS was mixed with 2 μL each of HEPES buffer alone or concentrated arecolone solutions prepared in the buffer, and fluorescence was measured after each addition of the solutions at the emission wavelength of 520 nm, at which an maximum fluorescence of ANTS was observed with an excitation wavelength of 370 nm. The relative fluorescence after each addition of arecolone solutions was subtracted from that of HEPES buffer, which was measured to account for the effect of ANTS dilution.

RESULTS

Stopped-Flow Studies of ANTS-Loaded Vesicles Using Arecolone, (+)-Epibatidine, and (±)-Epibatidine. Using ANTS-loaded membrane vesicles, arecolone, (+)-epibatidine, and (±)-epibatidine were examined for their activation properties of *Torpedo* nAcChR. Arecolone induced Tl^+ flux in a concentration-dependent manner with an activation constant (K_{act}) of 390 μM and a maximum flux rate (k_{max}) of 24 s^{-1} (Figure 1A and B). (+)-Epibatidine was a very potent agonist with $K_{act} = 19$ μM (Figure 1C). A concentration-dependent rate plot for (±)-epibatidine is shown in Figure 1D, where the value of K_{act} was estimated to be 39 μM by fitting data points with concentrations less than or equal to 100 μM (i.e., below saturation). At 500 μM, the rate was significantly decreased, possibly indicating inhibition at a high concentration by (−)-epibatidine or (±)-epibatidine. Normalization of the maximum rates of arecolone and (+)-epibatidine to Carb (100 s^{-1} at 320 μM, a convenient concentration for assays) resulted in 16 s^{-1} and 76 s^{-1}, respectively.

Effects of Eserine on PTS-Loaded nAcChR-Enriched Membrane Vesicles. Because previous studies with eserine used PTS, instead of ANTS, as a fluorescent dye to monitor

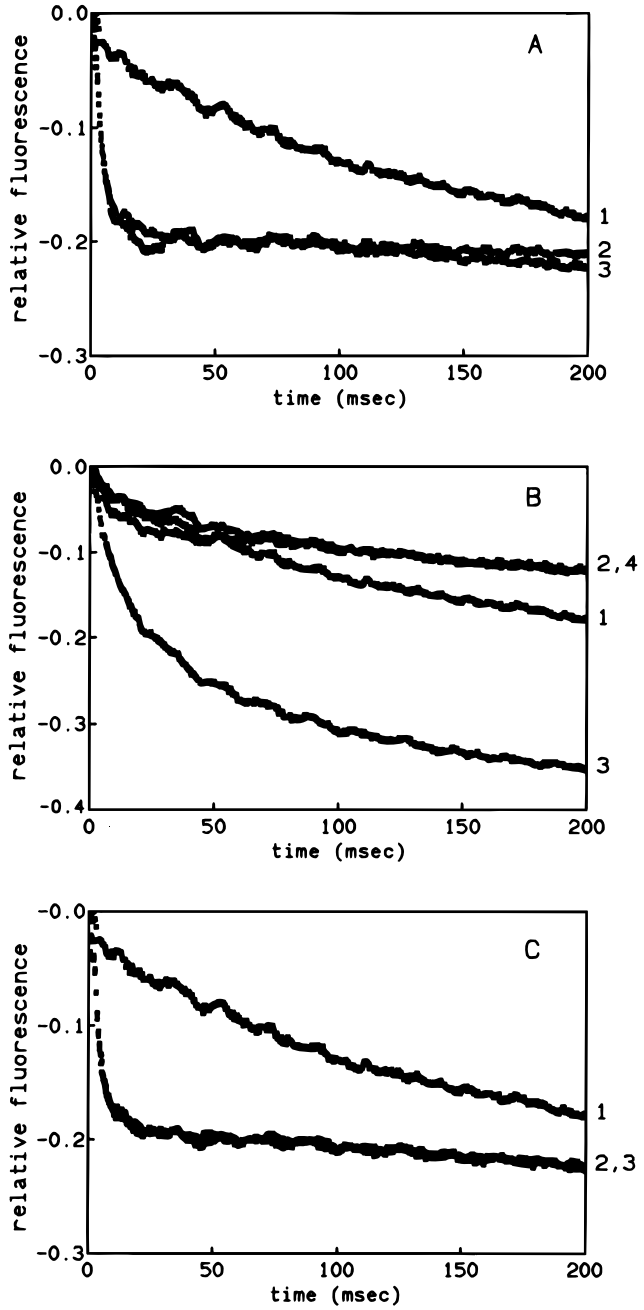

FIGURE 2: Eserine-induced stopped-flow responses with PTS-loaded nAcChR-rich membrane vesicles. (A) Response induced by 1 mM eserine in the presence (trace 2) or absence of Tl^+, which is replaced with Na^+ (trace 3). Trace 1 is Tl^+ leak. Traces were normalized so that the highest data point was zero for each trace. Each trace is an average of 2. (B) PTS-fluorescence quenching induced by 1 mM Carb in the presence or absence of 1 mM MTPP (trace 1) Tl^+ leak, (trace 2) 1 mM MTPP alone, (trace 3) 1 mM Carb, and (trace 4) 1 mM Carb and 1 mM MTPP. (C) Eserine-induced PTS-fluorescence quenching in the presence or absence of MTPP. (trace 1) Tl^+ leak, (trace 2) 1 mM eserine, and (trace 3) 1 mM eserine and 1 mM MTPP. All traces are averages of 3 or more traces.

the cation-flux response (*19, 20*), we initially studied PTS-loaded, nAcChR-enriched membrane vesicles. As previously observed, eserine concentration-dependent fluorescence quenching occurred and the trace for 1 mM eserine is shown in Figure 2A (trace 2). As a control, we replaced the fluorescence quencher, Tl^+, with Na^+, which does not quench the fluorescent dye, in the ligand-containing solution and observed an almost identical response (Figure 2A, trace 3), suggesting that the apparent activation response (fluorescence quenching) was not due to eserine-induced activation of the receptor and subsequent Tl^+ influx but was due to rapid fluorescence quenching of the dye by eserine itself. In our preparations, it was noted that the Tl^+ leak signal of PTS-loaded membrane vesicles was substantial compared with ANTS-loaded vesicles (see Figure 1, for example). This may be due to the structural nature of PTS, perhaps by having a nonspecific effect on the membrane.

We further examined whether eserine induced channel opening and flux of Tl^+ using a channel-blocking noncompetitive antagonist, MTPP (*25, 26*). When Carb was rapidly mixed with the vesicles in the presence of MTPP, Tl^+ flux was blocked as expected (Figure 2B, trace 4). However, the traces for eserine with or without the channel blocker were identical (Figure 2C, traces 2 and 3), further indicating that eserine-induced quenching of the dye was not due to Tl^+ transport. This suggested that the apparent "activation" signals were due to eserine itself, caused by interaction of the AcChE inhibitor with the dye, PTS, rather than agonist-induced cation transport.

Effects of Eserine on nAcChR-Rich Membrane Vesicles Loaded with ANTS. When *Torpedo* membrane vesicles loaded with ANTS were used for stopped-flow studies with eserine, the amine did not elicit fluorescence quenching in the concentration range 1 μM to 1 mM (Figure 3A). This demonstrated that eserine did not quench ANTS fluorescence and did not induce Tl^+ flux to cause quenching. As a control experiment, the stopped-flow studies were performed with Carb and eserine in the absence of Tl^+, which was replaced with Na^+. As expected from the results obtained in the preceding studies, no activation responses were observed for Carb or eserine (data not shown). When the flux buffer containing both eserine and various agonists (i.e., arecolone, (+)-epibatidine, and Carb) was rapidly mixed with the ANTS-loaded *Torpedo* membrane vesicles, eserine inhibited the agonist-induced response (Figure 3B and C). This inhibitory effect was concentration-dependent and yielded a similar inhibition constant for all agonists tested: IC_{50} = 150 μM using Carb, 150 μM using arecolone, and 160 μM using (+)-epibatidine. Therefore, in these concentration ranges, eserine acts as an antagonist rather than an agonist.

Interaction of Eserine with Fluorescent Dyes. To investigate the discrepancies of eserine action in comparing PTS- and ANTS-loaded membrane vesicles, the interaction of this ligand with the two fluorescent dyes was examined by obtaining equilibrium emission spectra of the dyes in the absence or presence of eserine (Figure 4). Eserine significantly quenched the fluorescence of PTS (12.5 mM) as a function of its concentration (Figure 4A). This fluorescence quenching was also observed at a PTS concentration of 125 μM (data not shown).

Eserine is a carbamate with a tertiary amine functionality with a pK_a of ~7.9. At pH 7.4, almost half of the ligand will be present as the deprotonated uncharged form which could readily traverse a lipid bilayer. It is possible that this deprotonated form of eserine could act as a quencher of PTS fluorescence. If such were the case, raising the pH from acid to more basic values would increase the extent of the PTS-fluorescence decrease by eserine with a half-maximal effect around the pK_a value. This was studied by varying the pH of the PTS and eserine solutions (Figure 4B). To assess the

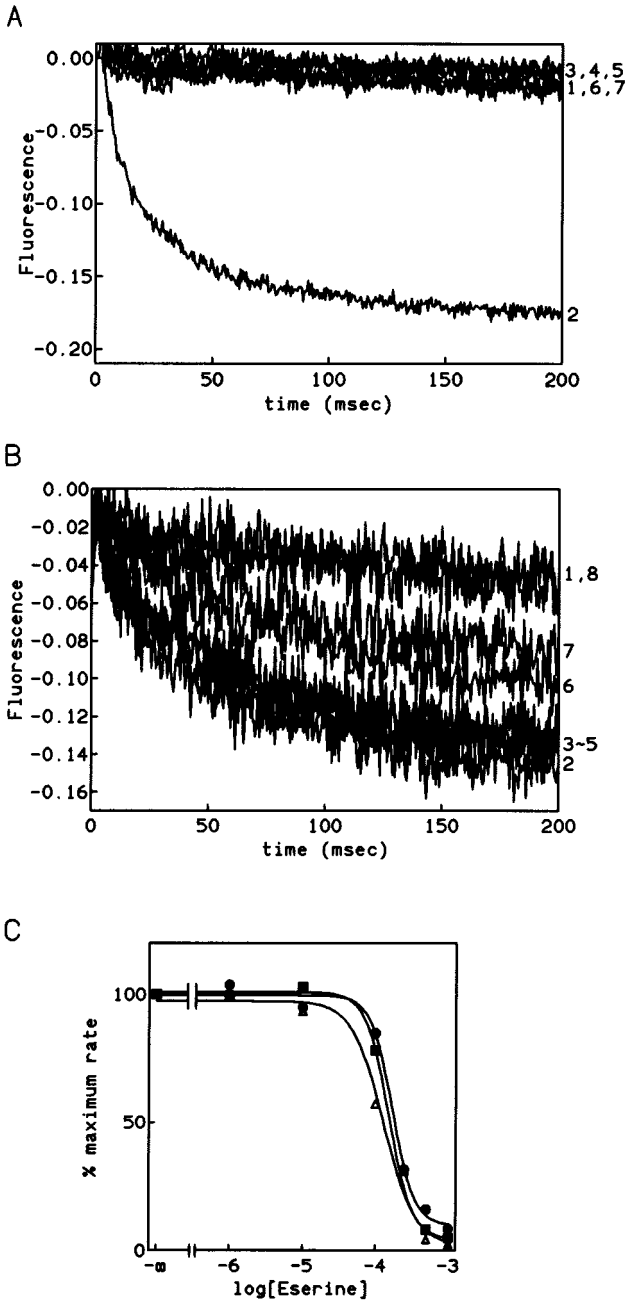

FIGURE 3: Effects of eserine on ANTS-loaded nAcChR-enriched membrane vesicles. (A) ANTS-loaded nAcChR-enriched *Torpedo* membrane vesicles were rapidly mixed with Flux buffer containing no (trace 1), 1 μM (trace 3), 10 μM (trace 4), 50 μM (trace 5), 500 μM (trace 6), 1 mM (trace 7) eserine. A trace for 1 mM Carb is also shown (trace 2). Initial points of all traces were normalized to zero. Each trace is an average of 3 or more traces. (B) Sample traces of ANTS fluorescence resulting from rapid Tl^+ influx induced by 10 μM (+)-epibatidine in the presence of the following concentrations of eserine: 0, 1 μM, 10 μM, 100 μM, 250 μM, 500 μM, and 1 mM (traces 2−8). Trace 1 is a control with only the Flux buffer and no ligand. (C) The concentration−response relationship for eserine inhibition of Tl^+ flux was induced by the following agonists: (△) 320 μM Carb, (●) 10 μM (+)-epibatidine, and (■) 1 mM arecolone. The rates of Tl^+ flux from at least 4 traces were averaged (standard error of the mean is not shown for clarity). IC_{50} of eserine for each agonist was as follows: 150 μM Carb, 160 μM (+)-epibatidine, and 150 μM arecolone.

extent of quenching, the difference in area between the fluorescence traces in the absence and presence of 1 mM eserine were estimated for each pH (Figure 4B, inset). The pH value at which eserine reduced the PTS fluorescence 50% was ~7.2, which is in reasonable agreement with the pK_a value, given the method used to estimate the difference in area (i.e., weighing the cutout traces; see legend for Figure 4).

The fluorescence of ANTS was not reduced by eserine in equilibrium fluorescence measurements (data not shown, but see traces in Figure 3A). To ensure that the activation responses obtained for arecolone, (+)-epibatidine, and (±)-epibatidine were not caused by their interaction with the loaded dye, we also obtained the emission spectra for ANTS in the presence of these amines. The extent of ANTS fluorescence quenched directly by arecolone (see Figure 1B) and (+)- and (±)-epibatidine (data not shown) was not substantial.

Stopped-Flow Studies with Lipid Vesicles Loaded with PTS. To confirm that eserine quenches PTS fluorescence within nAcChR-rich vesicles and produces the apparent concentration-dependent activation traces (i.e., fluorescence quenching) after crossing of the lipid bilayer, and to eliminate the possibility that eserine interacts with the receptor to activate it, lipids were extracted from *Torpedo* electric organ tissue and used to form vesicles devoid of the nAcChR. These lipid vesicles were loaded with the dye and were used in stopped-flow studies (Figure 5). In the absence of any proteins, including the nAcChR, eserine could still quench the fluorescence of PTS within the lipid vesicles, and its concentration dependence was qualitatively similar to data obtained with nAcChR-enriched membrane vesicles (Figure 5A; see also Figure 1A in *12*). This result supports the proposal that the uncharged form of eserine rapidly crosses the lipid bilayer and quenches the PTS fluorescence directly within vesicles. Replacement of Tl^+ with Na^+ in the ligand solutions resulted in essentially the same results, except that the leakage signal observed for Tl^+-containing solutions was abolished for Na^+-containing eserine solutions (comparing Figure 5A, trace 1 and Figure 5B, trace 1).

DISCUSSION

We investigated some tertiary amines for their effects on the *Torpedo* nAcChR using a stopped-flow Tl^+-flux system. Arecolone induced the flux of Tl^+ with a dissociation constant approximately 10 times larger than that of arecolone-methiodide, which was more potent than AcCh (unpublished observation). (+)-Epibatidine and (±)-epibatidine were very potent agonists with K_{act} values of 19 and 39 μM, respectively. The 2-fold increase in K_{act} for (±)-epibatidine compared with (+)-epibatidine can be explained by the presence of the levorotatory form of epibatidine in the enantiomeric mixtures, suggesting that the dextrorotatory form of epibatidine is considerably more potent than the levorotatory form in activating *Torpedo* nAcChR. The reduced rate for (±)-epibatidine at 500 μM (log[(±)-epibatidine] = −3.3; Figure 1D) suggests that (−)-epibatidine antagonizes (+)-epibatidine. The K_{act} of (+)-epibatidine was about 4.5 times lower than that of AcCh (87 ± 32 μM, unpublished data). This is in good agreement with a previous report that (+)-epibatidine was 3.5 times more potent than AcCh in activation of *Torpedo* nAcChR expressed in *Xenopus* oocytes (*11*). In contrast to these amines that activated the receptor, eserine was not an agonist for the nAcChR, in disagreement with previous reports (*12, 19, 20,*

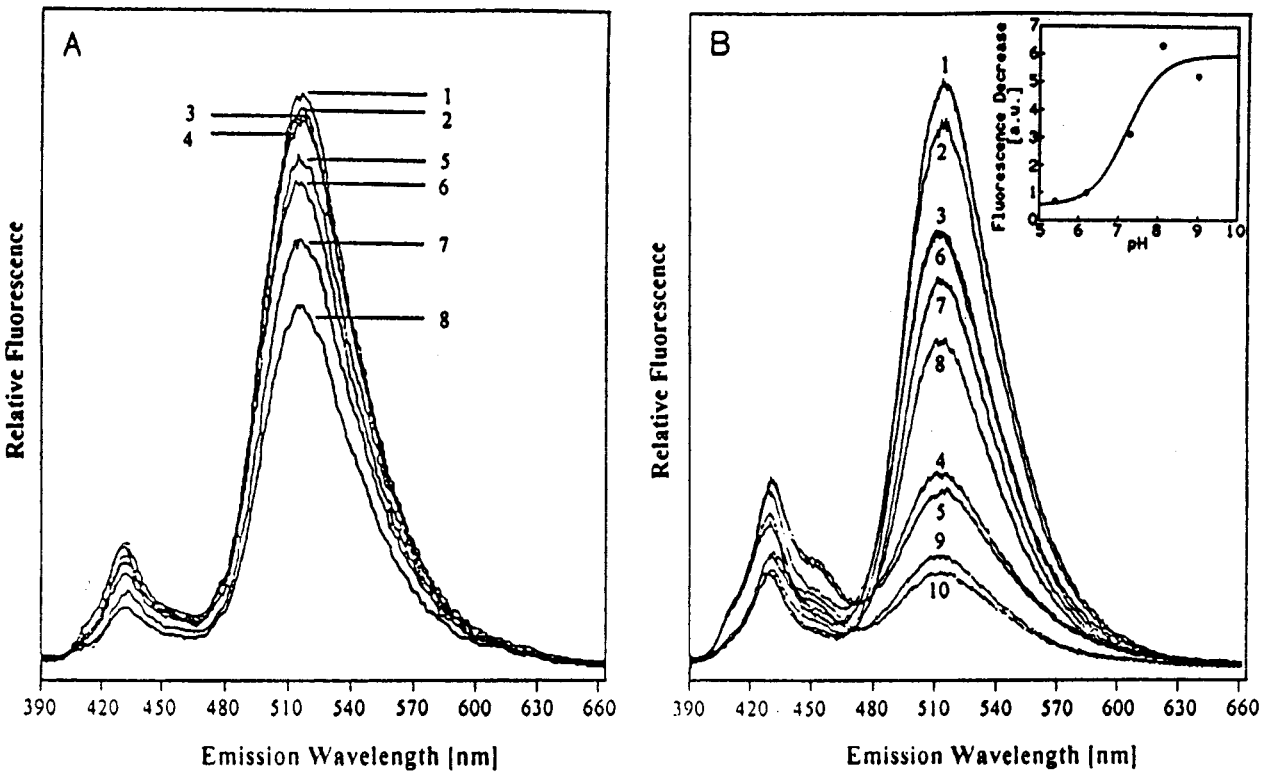

FIGURE 4: PTS fluorescence emission spectra with eserine. (A) The emission spectra of 12.5 mM PTS in the presence of 0 (PTS alone, trace 1), 31.2 μM (trace 2), 62.5 μM (trace 3), 125 μM (trace 4), 250 μM (trace 5), 500 μM (trace 6), 1 mM (trace 7), and 2 mM (trace 8) eserine. (B) PTS-fluorescence emission spectra at various pH values in the absence or presence of eserine. The PTS-fluorescence emission spectra were obtained without or with 1 mM eserine at pH 9.0 (traces 1 and 6), 8.1 (traces 2 and 7), 7.3 (traces 3 and 8), 6.2 (traces 4 and 9), 5.4 (traces 5 and 10), respectively. The order of fluorescence intensities below ~470 nm was reversed with those above ~470 nm (i.e., the intensity of trace 10 is highest before ~470 nm, whereas it is lowest after ~470 nm). Inset: The extent of quenching by eserine (i.e., fluorescent decrease) was estimated by cutting and weighing the two traces for each pH, and plotted as pH versus estimated weight. The points were fit to a sigmoidal curve. The pH at which the weight difference was 50% was 7.2.

27). Instead, it antagonized the agonist-induced activation responses in the *Torpedo* system studied here. Clearly factors other than the substitution level of the amine function are important in determining whether a given compound acts as an agonist or as an antagonist.

The studies represented here were undertaken to confirm and possibly extend work reported earlier describing a mechanism of *Torpedo* nAcChR activation by eserine distinct from that triggered by the natural neurotransmitter AcCh (*12*, *27*). Part of the evidence for a unique mechanism involved the observation that eserine could still activate the receptor even if it was desensitized by AcCh and blocked by α-bungarotoxin (α-BTx) or *d*-tubocurarine (*d*-Tc), and that the eserine activation traces were reduced to leakage traces by an antibody FK1, dibucaine, and benzoquinonium (*19*, *20*). These results were interpreted to indicate a novel action of eserine on the nAcChR, in which eserine activates the nAcChR through a second pathway distinct from that used by the natural neurotransmitter. We obtained similar data when the experiments were repeated using our system. Even if nAcChRs were desensitized or blocked by either α-BTx or *d*-Tc, eserine-induced apparent "activation" traces were still obtained.

However, the results presented here demonstrate that eserine does not induce ion flux in the *Torpedo* nAcChR-enriched membrane vesicles. Rather, eserine rapidly crosses the membrane and quenches the dye directly within the vesicles, resulting in apparent activation traces because (i) the nAcChR-enriched membrane vesicles loaded with ANTS, whose fluorescence was not reduced by the carbamate, did not result in any eserine-induced activation traces; (ii) the channel blocker MTPP, which inhibited the Carb-induced response, did not inhibit eserine-induced effects. If the channel path itself is blocked Tl^+ should not traverse the channel no matter by what pathway the receptor was activated; (iii) PTS-loaded lipid vesicles showed similar apparent "activation" traces in the presence of either Tl^+ or Na^+, (iv) eserine-induced, concentration-dependent apparent "activation" responses using the PTS-loaded lipid vesicles occurred with no receptor present. Therefore, the quench of fluorescence traces observed previously appear to be due to direct PTS fluorescence quenching by eserine itself.

With respect to this last observation, the direct interaction of eserine with PTS within the vesicle requires rapid transfer of the ligand through the lipid bilayer. The rapid equilibration of tertiary amine compounds across the membrane was considered to be responsible for their action on the channel block of voltage-gated sodium channels, where application of these amines from either inside or outside of the membrane blocked the ion flux (*28*). The rapid mixing of PTS-loaded lipid vesicles, with no nAcChR in their membranes, and eserine resulted in traces qualitatively similar to those obtained with membrane vesicles (see Figures 2A and 4), indicating the ability of eserine to cross the lipid bilayer on a millisecond time scale in the presence or absence of membrane proteins. This observation is not surprising considering the hydrophobic nature of the carbamate. The extent of PTS-fluorescence quenching by eserine depended

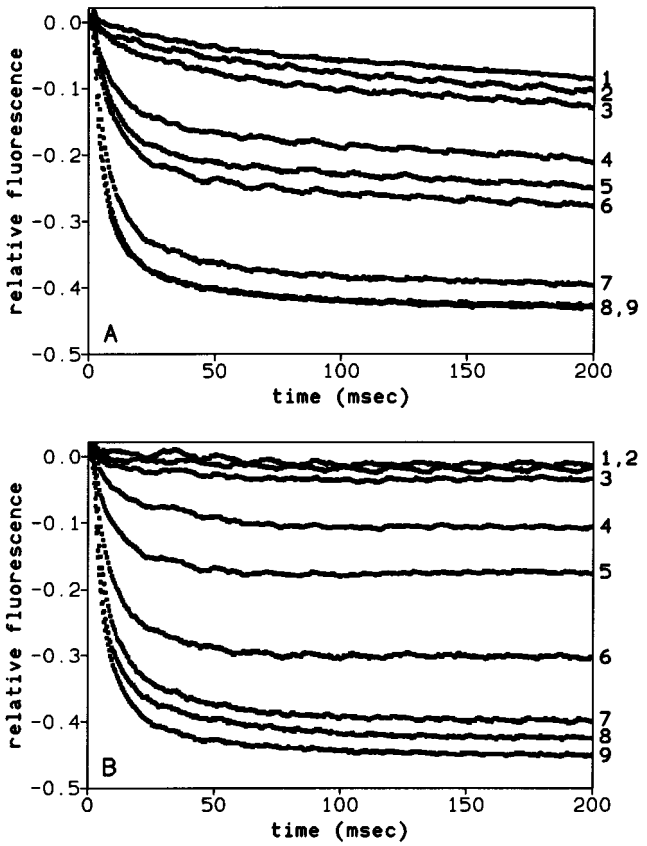

FIGURE 5: Stopped-flow fluorescence traces induced by eserine with PTS-loaded lipid vesicles. Kinetic traces of PTS fluorescence obtained after rapid mixing of PTS-loaded lipid vesicles and either Flux buffer (A) or Na^+ buffer (B) in the absence (trace 1) or presence of 1 μM (trace 2), 10 μM (trace 3), 50 μM (trace 4), 100 μM (trace 5), 250 μM (trace 6), 500 μM (trace 7), 1 mM (trace 8), 2 mM (trace 9) eserine. The highest point of each trace was normalized to zero. Each trace is an average of 3 or more traces.

on pH with quenching half-maximal at pH ~7.2, which is in reasonable agreement with the known pK_a of eserine. Furthermore, eserine methiodide, which contains a quaternary amine with a permanent positive charge, did not cause PTS-fluorescence quenching in the stopped-flow studies, although the fluorescence emission spectra of PTS measured at equilibrium were reduced by this compound (*29*). These observations support our contention that the uncharged form of eserine crosses the bilayer and quenches the loaded-dye PTS within the membrane vesicles.

Recent studies have shown that eserine can open single channels in frog muscle fibers at low concentrations and acts as a noncompetitive blocker at high concentrations (*15, 30, 31*). Similarly, benzoquinonium at low micromolar concentrations activated the receptor but blocked the channel at high concentrations as demonstrated by patch-clamp, single-channel studies on frog muscle fibers (*31, 32*). *d*-Tc, the classic nAcChR antagonist, also opened single channels of nAcChRs in rat myotubes (*33*) and of fetal-type receptors expressed on fibroblasts (*34*). Therefore, it is obvious that some ligands classically defined as antagonists can act as activators of the receptor at low concentrations and at the single-channel level. Although *d*-Tc was reported to induce currents in the fetal-type receptor expressed in fibroblasts (*34*), to our knowledge it has not yet been demonstrated that macroscopic currents can be elicited by eserine. The observations cited above in which certain cholinergic ligands activate receptors at low concentrations but block activation at high concentrations, cannot be accounted for by considering a single binding site or more than one equivalent site; rather, such effects necessitate the existence of binding sites or subsites (*35, 36*) that are nonequivalent.

We report here that eserine inhibited agonist-induced Tl^+-flux response with similar IC_{50} values for all agonists examined. The eserine concentrations that inhibited the activation response were within the concentration ranges reported to act as a channel blocker from electrophysiological studies. The IC_{50} value of ~150 μM we observed was near the value (200 μM) obtained for the study of inhibition of the indirect twitch tension of frog sartorious muscle (*30*), and the concentration profile of eserine inhibition on agonist-induced response was consistent with concentration-dependent depression of end-plate currents on the frog muscle (*15*).

With respect to specific eserine binding sites, eserine has been shown to inhibit the binding of [^{3}H]AcCh and [^{125}I]α-BTx at equilibrium (*37*), agreeing with results obtained in this laboratory, in which equilibrium [^{3}H]Carb binding was displaced by eserine and the rate of [^{125}I]α-BTx-nAcChR complex formation was inhibited by eserine (*29*). These data indicate that eserine competed at AcCh binding sites in desensitized receptors. In contrast to these findings, several studies indicated that eserine binds to a site(s) distinct from the agonist binding sites. Photoaffinity-labeling studies have demonstrated that the binding site for eserine lies in the region flanking Lys-125 of the α-subunit(s) in *Torpedo marmorata* AcChR, and the labeling was blocked by several ligands including antibody FK1 and benzoquinonium, but not by α-BTx (*38*). Studies with monoclonal antibodies FK1 and WF6 indicated that eserine binds to a sequence region distinct from the sites for AcCh, although within the same general region (*39*). In addition, eserine acts as a channel blocker at high concentrations, implying a direct interaction with the receptor (see above). Furthermore, a saturating NBD-fluorescence change on the NBD-labeled *Torpedo* nAcChR was induced by eserine in the presence or absence of α-BTx (*40*). It appears therefore that eserine can bind to the nAcChRs at a site distinct from agonist binding sites under equilibrium conditions. In the present article, we have ruled out the possibility that eserine induces macroscopic ion flux using *Torpedo* membrane vesicles.

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