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Direct spectroscopic studies of cation translocation by *Torpedo* acetylcholine receptor on a time scale of physiological relevance

(cation flux/functional polypeptides/single-channel transport/spectroscopic transport assay)

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ABSTRACT **The kinetics of carbamoylcholine-mediated cation transport across the membrane of vesicles containing acetylcholine receptor have been measured on the physiologically relevant time scale of a few milliseconds. The stopped-flow spectroscopic approach utilizes thallium(I) as the cation transported into sealed vesicles containing a water-soluble fluorophore. Upon entry of thallium(I), fluorescence quenching occurs by a heavy atom effect. Rapid thallium translocation into the vesicles is mediated by cholinergic agonists and is blocked by antagonists and neurotoxins and by desensitization. The kinetics of thallium transport are used to demonstrate that the four polypeptides known to comprise the receptor are the only protein components necessary for cation translocation. The kinetics of thallium(I) transport at saturating agonist concentrations are also used to calculate the apparent ion transport rate for a single receptor. The minimal value obtained is close to that for a single activated channel determined *in vivo*. This demonstrates that the physiological receptor has been isolated in intact form.**

Studies of the structure and function of the acetylcholine receptor (AcChoR) require quantitative methods to measure membrane permeability changes *in vitro*. Until now, filtration methods (1) have been used to assay ion efflux from AcChoR-enriched membrane vesicles using a radionuclide tracer (2), $^{22}Na^+$. The rate of efflux ($t_{1/2}$, ≈7 min) first reported was slower by approximately 5 orders of magnitude than that expected from electrophysiological measurements on intact cells in which conductance changes occur in 1–2 msec. Recently, more rapid $^{22}Na^+$ flux has been described (3–6) and the filtration method has been extended to the subsecond range, with the fastest samples corresponding to ≈25-msec time points (7).

We have developed a spectroscopic method to study AcChoR-mediated ion translocation on the time scale achievable by stopped-flow spectroscopy. This method involves rapid mixing, in a stopped-flow spectrometer, of a fluorescence quencher, thallium(I), with AcChoR-membrane vesicles entrapping a fluorophore. The Tl^+ ionic radius is similar to that of K^+ and it mimics K^+ in many membrane systems (8, 9). Moreover, Tl^+ is a strong fluorescence quencher, presumably due to the heavy atom effect (10, 11). By placing a fluorophore on one side and Tl^+ on the other side of the membrane, ion transport through AcChoR-associated channels can be studied by fluorescence quenching as Tl^+ moves across the membrane in response to an agonist on the physiologically relevant time scale of a few milliseconds. The results allow us to conclude that the AcChoR–protein complex of four homologous polypeptides (12) constitutes the physiological receptor in its entirety with its physiological functionality intact.

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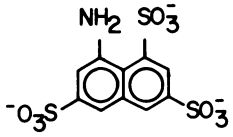
MATERIALS AND METHODS

AcChoR-enriched membrane vesicles were purified from *Torpedo californica* as described (6, 13). Unless otherwise specified, these membranes were routinely subjected to alkaline extraction (13, 14) to remove nonreceptor polypeptide contaminants as described (15).



ANTS

Membrane vesicles were loaded with the fluorescent probe 8-amino-1,3,6-naphthalenetrisulfonate (ANTS) as follows. One milliliter of 25 mM ANTS (Na salt) in 10 mM Na Hepes (pH 7.4) was mixed with 0.5 ml of membrane vesicles [20–40 μM α-bungarotoxin (α-BuTx) sites in 10 mM Na Hepes (pH 7.4)] at 4°C and quickly immersed in a liquid nitrogen bath for 3–5 min. The frozen mixture was thawed gradually at 0°C over a period of 0.5–1 hr. The "freeze–thaw" cycle was repeated twice more on the same mixture. External ANTS molecules were separated from the loaded vesicles by passing the mixture through a Sephadex G-25 column (1.7 × 24 cm, coarse resin) equilibrated with 35 mM $NaNO_3$/10 mM Na Hepes, pH 7.4, at room temperature. Membrane vesicles eluted in the void volume were collected and used without further dilution (1.2–1.9 μM α-BuTx sites).

Fast kinetic measurements of Tl^+ flux through the vesicle membrane were obtained by using a Durrum stopped-flow photometer (model D-110) set up in fluorescence mode and operating with a 75-W xenon lamp for excitation at 370 nm. Ion flux was initiated by rapidly mixing (machine dead time, ≈2.5 msec) a suspension of ANTS–vesicles with an equal volume of 35 mM $TlNO_3$/10 mM Na Hepes, pH 7.4, containing a nicotinic agonist or antagonist at 25 ± 1°C. Fluorescence emission was monitored by using a Corning C.S. 3-72 filter. The apparatus was connected to a Tektronix (Beaverton, OR) 5103N storage oscilloscope for visual monitoring of the data and to a Biomation (Sunnyvale, CA) model 805 transient recorder and a Digital Equipment Corporation (Maynard, MA) Minc computer for data storage and analysis.

Fluorescence spectra were recorded on a Perkin–Elmer MPF-4 fluorescence spectrometer. H_{10}-Histrionicotoxin (HTX) was a generous gift of Richard Cherpeck and D. Evans.

Abbreviations: AcChoR, acetylcholine receptor; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; α-BuTx, α-bungarotoxin; CbmCho, carbamoylcholine; HTX, histrionicotoxin; *d*-Tc, *d*-tubocurarine.

RESULTS

Choice of Fluorophore and Quenching by Thallium(I). Unlike most fluorophores, the highly charged, hydrophilic ANTS offers the advantages that it is impermeable to the vesicle membrane and it does not bind to or insert into the membrane bilayer significantly (data not shown).

ANTS exhibited broad excitation and emission spectra, with maxima at 370 nm and 515 nm, respectively. The fluorescence was effectively quenched by addition of Tl^+ (Fig. 1*A*) and the quenching gave a Stern–Volmer constant of 96 M^{-1} (Fig. 1*B*).

Loading of Fluorophore Within the Vesicles. Because of its low membrane permeability, ANTS was loaded into the AcChoR membrane vesicles by performing freeze–thaw cycles on membrane vesicles premixed with ANTS. Because the interior volume of the vesicles at concentrations appropriate for spectroscopic studies is only 1/5000th that of the bulk solution (unpublished data), exterior fluorophore molecules were removed by gel filtration to decrease background fluorescence. This procedure eliminated >99.9% of the exterior ANTS molecules from the loaded AcChoR vesicle preparation. The resulting vesicles are referred to as ANTS–vesicles. These vesicles exhibited fluorescence at 515 nm, indicating that ANTS was entrapped within them.

Kinetics of Thallium(I) Leak Across Vesicle Membranes Measured by Fluorescence Decay. When ANTS–vesicles were rapidly mixed with $TlNO_3$/Hepes buffer in a stopped-flow instrument, a slow fluorescence decay with a half-time of about 10 sec was observed (Fig. 2*A*). This decay represents quenching of the fluorescence by slow Tl^+ leakage through the resting membrane as judged by the following observations. (*i*) No fluorescence change was observed when $NaNO_3$/Hepes or KNO_3/Hepes was mixed with ANTS–vesicles (Fig. 2B). (*ii*) The slow kinetics of fluorescence decay were absent when vesicles with ANTS present only on the outside of the membranes were mixed with $TlNO_3$/Hepes; instead, the fluorescence dropped instantaneously (within the mixing time of the instrument) to the equilibrium quenched level. Similarly, the slow kinetic signal was abolished by disruption of the ANTS–vesicle membranes by Triton, phospholipase, sonication, or osmotic shock.

Kinetics of Thallium(I) Influx in the Presence of Cholinergic Agonists. Addition of carbamoylcholine (CbmCho) to the $TlNO_3$/Hepes buffer greatly enhanced the rate of fluorescence decay when the latter was mixed with ANTS–vesicles. The lower traces in Fig. 3 *A* and *B* demonstrate the effect produced by 100 μM CbmCho. The fluorescence decayed rapidly with a half-time of about 30 msec. The slow leakage of Tl^+ through the resting membranes described in the preceding paragraph ($t_{1/2}$, ≈10 sec) was negligibly small on this fast time scale (Fig. 3 *A* and *B*, top traces). Acetylcholine also markedly increased the rate of fluorescence decay. Activation of AcChoR-associated channels by nicotinic agonists therefore increased the permeability of the vesicle membrane to Tl^+ and the kinetics of ion transport could be studied by measuring the rate of quenching of the ANTS fluorescence.

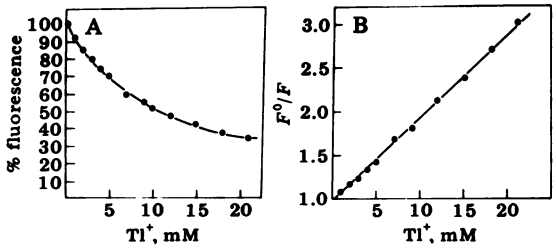


FIG. 1. Quenching of ANTS fluorescence by Tl^+. (*A*) Fluorescence of 165 μM ANTS in the presence of increasing concentrations of $TlNO_3$ was measured at excitation = 370 nm and emission = 515 nm. The ionic strength of the solutions was kept constant by replacing $TlNO_3$ with $NaNO_3$ (total concentration, 35 mM), and the solutions were buffered at pH 7.4 with 10 mM Hepes. (*B*) Stern–Volmer plot of the data in *A*. F^0 and F are the fluorescence intensities in the absence and presence of Tl^+, respectively. The solid line indicates a least-squares fit of the data to a straight line, with a slope of 96 M^{-1} and a correlation coefficient of 0.998.

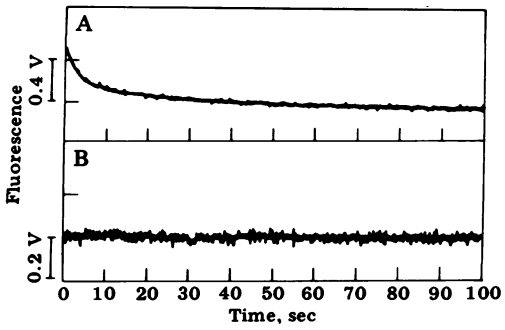


FIG. 2. Rate of Tl^+ leakage through the vesicle membrane in the absence of agonist. (*A*) Membrane vesicles loaded with ANTS (syringe 1) were mixed with 35 mM $TlNO_3$/10 mM Na Hepes at pH 7.4 (syringe 2) in a stopped-flow instrument and the fluorescence was monitored. (*B*) Control experiment with $TlNO_3$ replaced by $NaNO_3$.

Pharmacological Effects on Thallium Influx Kinetics. In the presence of 1 mM *d*-tubocurarine (*d*-Tc), the fast fluorescence decay produced by 100 μM CbmCho was completely inhibited (Fig. 3*D*) and the antagonist by itself did not produce any detectable fluorescence change (Fig. 3*C*). HTX, an ion conductance-perturbing agent (16, 17), abolished the CbmCho-induced signal when present at a concentration of 10 μM (Fig. 3*F*) but had no effect on the fluorescence in the absence of CbmCho (Fig. 3*E*). Preincubation of the AcChoR vesicles with excess α-BuTx also blocked the effect produced by CbmCho (Fig. 3*H*); the α-BuTx alone had no effect on the fluorescence (Fig. 3*G*). ANTS–vesicles preincubated with 100 μM CbmCho at room temperature for 30 min did not exhibit any fast fluorescence decay when mixed with $TlNO_3$/Hepes containing either 100 μM or 0 μM CbmCho (Fig. 3 *I* and *J*). Activation of the permeability change was thus inhibited as a result of receptor desensitization.

These pharmacological tests support the notion that the fluorescence signal produced by cholinergic agonists indeed represented rapid (millisecond) ion flux through AcChoR-activated ion channels. The use of this method to solve two fundamental problems related to AcChoR function is presented below.

Rate of Thallium(I) Influx as a Measure of Functional AcChoR Components. In general, the rate of ion flux through the membrane vesicles is proportional to the number of activated channels present on the membrane surface (15, 18) (also see *Appendix*). The rate of fluorescence decay produced by cholinergic agonists therefore serves as a measure of the active AcChoR population. The effect of a given treatment or modification on function of the AcChoR can be examined by comparing the rates of agonist-specific Tl^+-induced fluorescence decay under the same conditions in a preparation before and after the modification. One such example is illustrated in Fig. 4 where the transport kinetics of an AcChoR preparation were compared before and after alkaline extraction (13, 14) to remove at least 98% of the M_r 43,000 and M_r 90,000 polypeptides (15). These alkali-extracted vesicles contained only the four AcChoR polypeptide subunits (15). The apparent rates (see

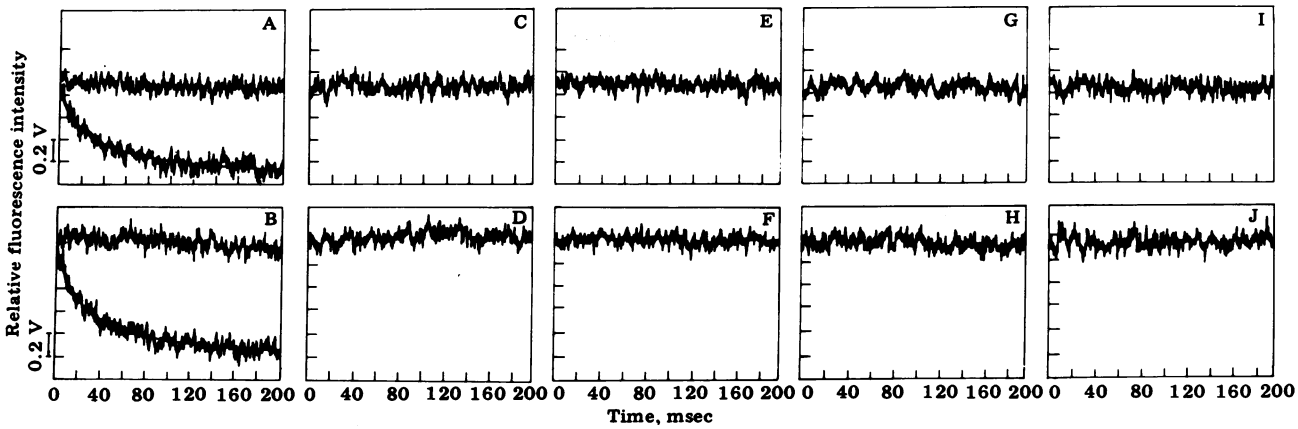


FIG. 3. Kinetics of agonist-induced Tl^+ influx through AcChoR–membrane vesicles and its blockage by cholinergic antagonists and by desensitization. (*A*) Membrane vesicles entrapping ANTS (syringe 1) were rapidly mixed with $TlNO_3$/buffer with (lower trace) or without (top trace) 200 μM CbmCho (syringe 2) and the fluorescence was recorded. Final concentration of CbmCho was 100 μM. (*B*) Same as in *A* with a different membrane preparation. Note line in *A* and *B* indicating least-squares fit of the data to Eq. 1 (see *Appendix*), yielding k_{app} = 20 and 17 s^{-1} for *A* and *B*, respectively. (*C* and *D*) Effect of *d*-tubocurarine (*d*-Tc). Syringe 1 contained ANTS–vesicles; syringe 2 contained 2 mM *d*-Tc in $TlNO_3$/buffer (*C*) or 2 mM *d*-Tc plus 200 μM CbmCho in $TlNO_3$ buffer (*D*). (*E* and *F*) Effect of HTX. Syringe 1 contained ANTS–vesicles preincubated with 10 μM HTX for 5 min; syringe 2 contained 10 μM HTX in $TlNO_3$/buffer (*E*) or 10 μM HTX plus 200 μM CbmCho in $TlNO_3$/buffer (*F*). (*G* and *H*) Effect of α-BuTx. Syringe 1 contained ANTS–vesicles preincubated with excess α-BuTx; syringe 2 contained $TlNO_3$/buffer (*G*) or 200 μM CbmCho in $TlNO_3$/buffer (*H*). (*I* and *J*) Effect of desensitization. Syringe 1 contained ANTS–vesicles preincubated with 100 μM CbmCho for 30 min; syringe 2 contained $TlNO_3$/buffer (*I*) or 100 μM CbmCho in $TlNO_3$/buffer (*J*).

legend to Fig. 4) measured under the same conditions were within a factor of 2 of each other. This result thus independently substantiates the idea (5, 15) that the M_r 43,000 and M_r 90,000 polypeptides, or indeed any polypeptide other than the four receptor subunits of M_r 40,000, 50,000, 60,000, and 65,000, are nonessential for AcChoR-mediated ion translocation. In accord with our previous observations using $^{22}Na^+$ flux measurements, the flux amplitude of the untreated preparation was smaller than that of the treated membranes, indicating that the former contained a population of contaminating vesicles lacking AcChoR.

Ion Transport Rate Mediated by a Single AcChoR Molecule. An important question is whether the AcChoR isolated *in vitro* represents functionally intact, unimpaired receptor.

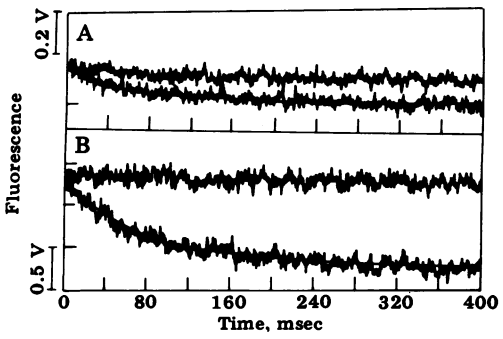


FIG. 4. Comparison of Tl^+ influx produced by 100 μM CbmCho with untreated AcChoR membranes (*A*) and alkali-extracted membranes (*B*). An AcChoR-enriched membrane preparation was divided into two halves. One was subjected to alkaline extraction and the other was treated in parallel but the pH was kept at 7.4. Both were loaded with ANTS and eluted from the gel filtration column with 70 mM $NaNO_3$/1 mM Na Hepes, pH 7.4. Stopped-flow measurements were carried out with syringe 1 filled with ANTS–vesicles and syringe 2 filled with 70 mM $TlNO_3$/1 mM Na Hepes at pH 7.4 (upper trace) or the same solution containing 200 μM CbmCho (lower trace). Least-squares fits of the data to Eq. 1 gave k_{app} = 8 and 5 s^{-1} for the untreated and treated vesicles, respectively. Observed rates differed by a factor of 2–3 from those in Fig. 3 due to use of buffers of different ionic composition.

This can be approached by determination of the number of ions transported per second through each activated channel and comparison to the known single-channel conductance measured *in vivo*. In the absence of detailed mechanisms for channel activation and inactivation, this number could be estimated by measuring the full flux rate at high CbmCho concentrations and dividing it by the total number of AcChoR molecules present in each vesicle. This yields the lower limit of the actual transport number, because an extreme situation was assumed in which all the channels present were considered to be open at saturating CbmCho concentrations throughout the time course of the flux experiments.

Fig. 5*A* illustrates the signals produced by increasing concentrations of CbmCho; the rate of fluorescence decay increased dramatically as the concentration of CbmCho was raised from 0 to 500 μM. At CbmCho concentrations higher than 500 μM the kinetics were too fast to be measured by the stopped-flow instrument. To estimate the full flux rate at high CbmCho concentration, titration with CbmCho in this range was carried out in the presence of 1–2 μM HTX to partially inactivate the AcChoR and thereby reduce the flux rate. In different experiments it was found that addition of 1–2 μM HTX slowed the flux rate by a factor of 4–6 at 100 μM CbmCho without appreciably affecting the amplitude. For each preparation this factor was determined and used empirically to normalize the flux rates measured in the presence of HTX at higher CbmCho concentrations (Fig. 5*B*). At the maximal rate ($\approx$1500 sec^{-1}) extrapolated from the dose–response curve, a minimal transport number of 10^6 ions per sec per activated channel was calculated (see *Appendix*). This is within 1 order of magnitude of that reported for AcChoR-activated channels in intact cells [$\approx 10^7$ sec^{-1} (19–21)]. The AcChoR complex in the isolated preparations therefore closely resembles that in intact cells, and a large fraction of the AcChoR is apparently fully functional in our purified membranes.

The dependence of the flux rate on CbmCho concentration was fitted to a simple model which assumed either one or two agonists to bind for channel activation, (Fig. 5 *B* and *C*). Both models fit equally well and cannot be differentiated based on the current data.

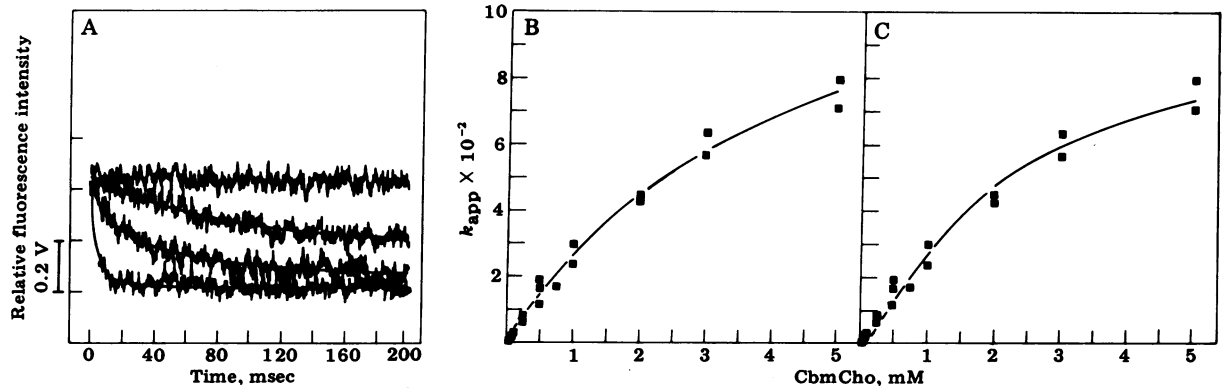


FIG. 5. Dependence of the observed flux rate on the concentration of CbmCho. (*A*) Kinetics of Tl^+ influx produced by 0, 50, 100, and 500 μM CbmCho (top to bottom). Solid lines indicate computer-fitted curves to Eq. 1. (*B* and *C*) Plot of the apparent flux rate constant as a function of the concentration of CbmCho. Data were obtained from three different preparations. The kinetic traces were fitted to Eq. 1 and k_{app} was determined. The rate constants at concentrations of CbmCho higher than 500 μM were obtained by carrying out the flux experiments in the presence of 1–2 μM HTX and normalizing the values by a factor predetermined at 100 μM CbmCho for the given preparation to give those in the absence of HTX. The normalization procedure assumed a constant percentage of blockage of the flux rate at all agonist concentrations by a given concentration of HTX. The data were fitted to binding isotherms by assuming either one (*B*) or two (*C*) CbmCho molecules were associated with each activated channel [$k_{app} = k_{max}/(1 + (K_d/[L]))$ for *B* and $k_{app} = k_{max}/(1 + (K_d/[L]))^2$ for *C*], yielding $k_{max} \approx 1500$ and $K_d \approx 5$ mM for *B* and $k_{max} \approx 1100$ and $k_d \approx 1$ mM for *C*.

DISCUSSION

For quantitative analysis of ion transport processes across vesicle membranes *in vitro* it is highly desirable to have the high information content of a continuous recording method such as an optical signal, especially to facilitate computer-aided analysis of the data and ultimately for dissection of reasonable mechanisms. Spectroscopic approaches for such studies, however, encounter many difficulties because the internal volume of the vesicles represents a very small fraction of the total. We have overcome such problems by loading the optical probe within the vesicles and measuring ion influx. As noted in Fig. 3, signals as large as $\approx$1.0 V can be generated by this method. The first use of this method has been to show that only the four AcChoR polypeptides are essential for cation translocation. Direct comparison of the ion flux kinetics from an untreated AcChoR preparation and one lacking the M_r 43,000 and M_r 90,000 polypeptides after alkaline extraction (13–15) showed striking similarities (Fig. 4). These polypeptides are thus nonessential for the AcChoR-mediated ion translocation and cannot represent ion channel components, consistent with previous findings by partial inactivation (15) and by reconstitution (5).

The slow ion flux first reported by Kasai and Changeux (1) could be interpreted to suggest that loss of functional elements during the isolation procedures was not unlikely. Although better preparative methods have been used and preparations that exhibited ion flux on a faster time scale have been obtained (3–6), the complete functional integrity of receptor preparations could not be unequivocally determined due to the lack of available techniques to measure ion flux kinetics on rapid time scales. We estimate that at least 10^6 ions were transported through each AcChoR molecule per sec in our purified preparations (see *Appendix*). This number is a minimal estimate because all the channels were assumed to be open at all times throughout the flux experiment at saturating concentrations of agonist. Because the probabilities for channel opening when agonists are bound are considered to be less than 1 (22), this implies that the actual transport number should be higher than calculated. This and the differences in membrane potential and ion composition may therefore adequately account for the difference between the transport number we obtain and that reported for the intact AcChoR *in vivo* [$\approx 10^7$ sec^{-1} (19–21)]. Our purified AcChoR preparations thus closely resemble the native receptor in intact cells.

The flux rates we observe by using *Torpedo* membrane vesicles are much faster than those reported recently by Hess *et al.* (7) with eel vesicles [half-time, $\approx$160 msec at 1 mM CbmCho, compared to 2.5 msec determined here (Fig. 5)]. Whether this discrepancy is only due to differences in the AcChoR densities in the two species or in the functional integrity of the two preparations awaits further clarification.

The observed dependence of the flux rate on the concentration of agonists showed that it did not saturate at concentrations expected for binding of agonists to the low-affinity site of the AcChoR (23). Instead, the rate began to level off only at high concentrations of CbmCho (millimolar range). This implies that a scheme more complicated than the simple occupancy mechanism for this low-affinity site may be involved in channel activation. Quantitative analysis of the flux kinetics coupled with fluorescence studies of AcChoR-associated conformational changes (24) should reveal a detailed mechanism for activation and inactivation of the AcChoR-associated channels. In addition, because Tl^+ can be substituted for K^+ in many biological systems, the Tl^+ quenching technique presented here can be applied to study of ion transport through other excitable membranes *in vitro*.

APPENDIX

Quantitative analysis of the kinetics of the optical signal can be achieved by combining the rate law of Tl^+ influx and the Stern–Volmer relationship for fluorescence quenching. Under conditions in which the number of activated channels is constant during the flux process, the kinetics of Tl^+ influx can be described by a first-order process: $T(t) = T_\infty(1 - e^{-kt})$ in which T is the concentration of Tl^+ inside the vesicles at time t, and k is the apparent rate constant of Tl^+ flux which is a complex function of channel conductance, vesicle size, surface density of channels, ion composition, and the agonist concentration (15). The fluorescence of ANTS at a given internal Tl^+ concentration T is given by the Stern–Volmer relation $F^0/F = 1 + KT$ (see Fig. 1) in which K is the Stern–Volmer constant. Substituting T into the above expression, one obtains the fluorescence intensity as a function of time $F = F^0/[1 + KT_\infty(1 - e^{-kt})]$ (Eq. 1).

By fitting the kinetic traces to this equation, apparent rate

constants for Tl^+ flux were determined (see Fig. 3). The minimal number of ions transported through each activated channel was estimated as follows. For each vesicle, the rate of unidirectional flux of Tl^+ is the concentration of Tl^+, C, multiplied by the apparent flux rate constant, k. Under the experimental conditions used, C is 17 mM (concentration of Tl^+ outside the vesicles) and k is $\approx$1500 from the empirically fitted flux rate (Fig. 5). The rate of entry of Tl^+ is therefore $\approx$26 M/sec. Taking into consideration the interior volume of the vesicles [with an average diameter of 6000 Å (13), volume is $\approx 10^{-16}$ liter/vesicle], this means that $26 \times 10^{-16} \times 6.02 \times 10^{23}$ or $\approx 2 \times 10^9$ Tl^+ ions entered each vesicle per second when the surface AcChoRs were activated. The number of AcChoR oligomers per vesicle calculated from the reported density [$\approx 10^4/\mu m^2$ (25, 26)] and the surface area of vesicles 6000 Å in diameter is $\approx 10^4$. The minimal number of Tl^+ ions transported per second through each activated AcChoR channel is therefore (see *Discussion*) 2×10^5. Because other permeant ionic species were present, the transport number for Tl^+ should be corrected to give the total transport number for bidirectional flux of all ionic species (summation of the total concentrations of Na^+ and Tl^+ on both sides of the bilayer gave 104 mM). If Na^+ and Tl^+ have similar permeabilities, at least 10^6 ions were transported through each activated channel per second. For *Torpedo* Ringer's solution (equivalent to 400 mM NaCl) this value becomes (assuming linearity) $\approx 7 \times 10^6$ ions sec^{-1}.

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