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Quantitation of cation transport by reconstituted membrane vesicles containing purified acetylcholine receptor

(reconstitution/cation flux/single-channel transport/stopped-flow spectroscopy)

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ABSTRACT A stopped-flow spectroscopic technique was used to study the kinetics of ion transport by reconstituted membrane preparations containing purified acetylcholine receptor. Influx of thallium (I) into membrane vesicles was monitored as a decrease, due to quenching by the thallous ion, in the fluorescence of an entrapped fluorophore. In a reproducible manner, the reconstituted receptor responded to cholinergic agonists by mediating rapid ion transport in the millisecond time range. The effect of agonists was blocked by receptor desensitization and by histrionicotoxin and was absent in membrane vesicles lacking receptor. Analysis of the fast kinetics of cation transport produced by saturating concentrations of agonists yielded an estimated rate of transport through a single reconstituted receptor channel. Comparison of this rate with those reported for *in vivo* preparations and for purified membranes shows that the reconstituted protein closely resembles the physiologically active receptor.

The acetylcholine (AcCho) receptor (AcChoR) from *Torpedo californica* electroplax is a pentamer composed of two identical and three related and homologous polypeptides (1) and having M_r 255,000 based on the stoichiometry of the subunits (1, 2). Membrane preparations containing no protein components other than the receptor complex have been shown to bind agonists (3, 4), antagonists (5, 6), and neurotoxins (7–9), as well as a local anesthetic analogue (10) and histrionicotoxin (HTX) (10, 11). In addition, such preparations respond to cholinergic agonists by mediating cation translocation into or out of the interior of the membrane vesicles (10, 12). Studies of this cation translocation using both slow (13) and fast (14) kinetic methods have led to the conclusion that this single molecular complex is the only protein component necessary for full function in terms of cation transport. Preliminary reports of cation transport by reconstituted preparations derived from purified AcChoR (15–18) have shown qualitatively that the AcChoR complex is capable of conducting cation transport. However, these studies have not allowed the quantitation of cation translocation necessary for determination of whether the reconstituted preparations retain the full physiological properties of the receptor *in vivo*.

Recently, we developed a spectroscopic method that allows study of ion translocation on the time scale achievable by stopped-flow spectroscopy; i.e., a few milliseconds (14). In the work reported here, we used this method to resolve the kinetics of ion transport across reconstituted AcChoR-containing membrane vesicles. Quantitative analysis of the fast ion-flux kinetics yielded an AcChoR-mediated transport rate that is comparable with that reported for *in vivo* preparations.

MATERIALS AND METHODS

Preparation of Purified AcChoR-Containing Membranes. Highly purified AcChoR-containing membranes were obtained as described (19, 20). These preparations have been shown to contain only the four polypeptides of the AcChoR on the basis of criteria such as $NaDodSO_4$/polyacrylamide gel analysis (19, 20) and NH_2-terminal amino acid sequence analyses (1).

Solubilization and Reconstitution of AcChoR. The AcChoR protein was extracted from the purified membranes and reconstituted as described (15, 20) with the following modifications. Purified membranes were solubilized in Na buffer (10 mM Na Hepes, pH 7.4/200 mM $NaNO_3$ containing 2% Na cholate and sonicated asolectin at an asolectin/protein ratio of 2:1. After removal of nonsolubilized material by centrifugation at 140,000 × *g* for 1 hr, sonicated asolectin was added to a final asolectin/protein ratio of 10:1. The protein/lipid mixture was stirred for 15 min and centrifuged at 140,000 × *g* for 30 min. Detergent was removed by dialysis against 1000 vol of Na buffer at room temperature for 20 hr.

Loading of Reconstituted AcChoR-Containing Vesicles with Fluorophore. The fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) was purchased from Chemical Service, West Chester, PA. It was loaded into reconstituted vesicles by the freeze-thaw procedure (14), except for the following changes: 0.5 vol of 25 mM ANTS in 10 mM Na Hepes, pH 7.4/125 mM $NaNO_3$ was mixed with 1 vol of reconstituted membrane vesicles at room temperature. The mixture was frozen and kept at −90°C for 30 min and then allowed to thaw gradually at room temperature, usually for 1.5–2 hr. Removal of external ANTS was achieved by passing the thawed mixture through a 1.7 × 24 cm Sephadex G-25 column. Reconstituted AcChoR-containing vesicles loaded with ANTS (reconstituted ANTS vesicles) were collected in the void volume and used for cation flux studies without further dilution.

Quantitation of α-Bungarotoxin Sites and of Phospholipids in Reconstituted Preparations. Reconstituted ANTS vesicles were assayed for ^{125}I-labeled α-bungarotoxin (α-BuTx) binding sites (0.22–0.31 μM) according to published procedures (21). The phospholipid content of the same vesicle preparation (3 mM) was determined by a modified version (22) of the total phosphorus assay of Fiske and SubbaRow, except that a combination of 1.25 M H_2SO_4 and 10% HNO_3 was used in place of $HClO_4$ during lipid digestion.

Stopped-Flow Measurements of Thallium Transport into Reconstituted AcChoR Vesicles. Tl^+ transport in reconstituted preparations was measured in a stopped-flow instrument as described (14). Tl^+ flux was initiated by rapid mixing at 25°C of a reconstituted ANTS-vesicle suspension and an equal volume of 28 mM $TlNO_3$ in 10 mM Na Hepes, pH 7.4/172 mM $NaNO_3$ containing a nicotinic agonist and, in some cases, HTX. Fluorescence emission was monitored and analyzed as reported (14). HTX was a generous gift of R. Cherpeck and D. E. Evans.

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; ANTS, 8-amino-naphthalene-1,3,6-trisulfonate; α-BuTx, α-bungarotoxin; CarbCho, carbamoylcholine; HTX, histrionicotoxin.

RESULTS

Kinetics of Thallium Influx Across Reconstituted Membrane Vesicles Containing Purified AcChoR. Transport of Tl^+ into vesicles was studied by measuring the decrease in ANTS fluorescence due to quenching by Tl^+ (Fig. 1). When reconstituted ANTS vesicles were rapidly mixed with Tl^+ in a stopped-flow instrument, a fluorescence decay with a half-time of ≈0.9 sec was observed (data not shown). This decay presumably represents leakage of Tl^+ through the membrane and into the interior of the vesicles. Addition of 1 mM carbamoylcholine (CarbCho) in the $TlNO_3$/Hepes buffer resulted in a marked acceleration of this fluorescence decay (Fig. 2 *A* and *B*). This rapid fluorescence decay had a half-time of about 5 msec. At significantly lower concentrations, AcCho had a similar effect (Fig. 3*B*) on the fluorescence decay. Thus, activation of the ion channel of the AcChoR greatly enhanced the rate of influx of Tl^+. These results show that the reconstituted membranes containing purified AcChoR retain the capability to induce cation permeation in response to nicotinic agonists on a physiological time scale.

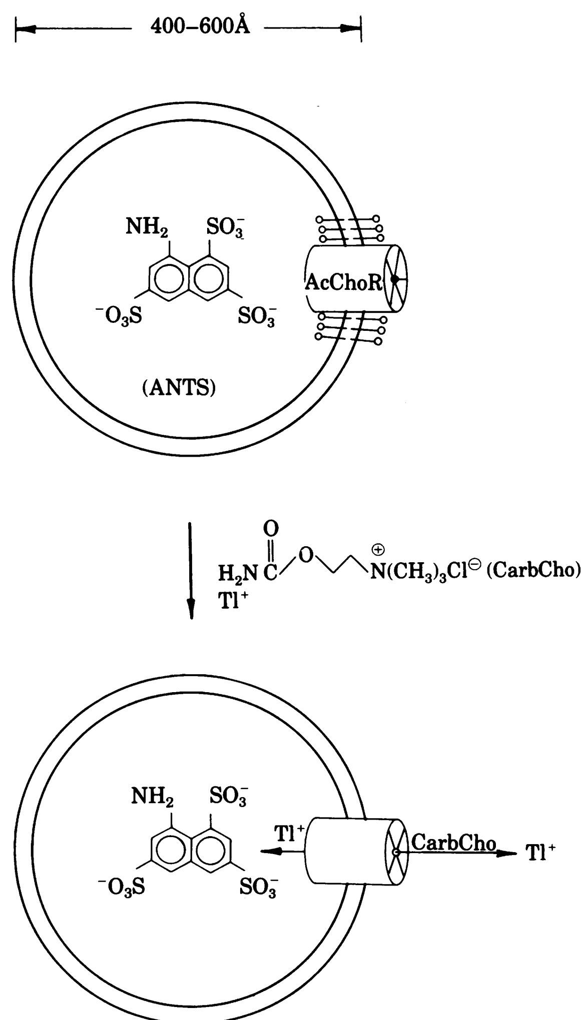

FIG. 1. Schematic diagram depicting fluorophore entrapped in reconstituted AcChoR vesicles. (*Upper*) On addition of a cholinergic agonist and Tl^+ to the vesicle exterior, the receptor channel opens (*Lower*) to allow entry of Tl^+ (all cations present may respond via a simple bidirectional equilibration). Fluorescence emission by the entrapped fluorophore is instantaneously quenched by Tl^+.

Several observations suggest that the rapid enhancement of Tl^+ influx produced by CarbCho was a specific effect. Tl^+ leakage into liposomes lacking AcChoR had a half-time of about 0.5 sec, and this was not altered by addition of agonist (see Fig. 2 *G* and *H*). The data in Fig. 2 *C* and *D* show that the CarbCho-activated acceleration of fluorescence decay was completely abolished by 10 μM HTX, a toxin reported to possibly interact with AcChoR-associated ion channels (23, 24). In addition, desensitization resulting from preincubation of reconstituted AcChoR-containing vesicles with 100 μM CarbCho inhibited the rapid fluorescence decay (Fig. 2 *E* and *F*) produced by 1 mM CarbCho. These results show that the reconstituted preparations manifest the pharmacological properties of the AcChoR on the physiologically significant time scale.

Rate of Ion Transport Through a Single Reconstituted AcChoR Molecule. An important question concerning the reconstituted membranes is whether the receptor has preserved its full functional integrity with respect to its capability to mediate the very high rate of agonist-activated ion transport recorded *in vivo* (25–27). Accordingly, the rate of ion transport per vesicle at saturating concentrations of CarbCho was estimated by dividing the observed full flux rate by the average number of receptor molecules per vesicle to obtain the number of ions transported through a reconstituted receptor channel per second.

Flux experiments carried out at higher concentrations of CarbCho or AcCho gave fluorescence traces with higher decay rates. At concentrations of CarbCho or AcCho >1 or 40 μM, respectively, the initial phase of the cation transport kinetics was too fast to be measured by the stopped-flow instrument. Activation of ion transport by high concentrations of agonists was therefore performed in the presence of 1 μM HTX to randomly inactivate a fraction of the receptors and thereby slow the flux rates to measurable levels. By using different reconstituted preparations, it was found that inclusion of 1 μM HTX with 10 or 20 μM AcCho or 400 μM CarbCho reduced the flux rates by a factor of ≈2. Thus, the apparent rate constants for Tl^+ flux obtained for agonist concentrations of CarbCho or AcCho >750 or 30 μM, respectively, in the presence of 1 μM HTX were normalized to the expected control values (without HTX) by applying this factor. Fig. 3 *C* and *D* shows the apparent flux rates determined for different concentrations of CarbCho and AcCho. The rates for CarbCho began to level off at concentrations in the millimolar range. When these data were fitted to a binding isotherm that assumed association of two CarbCho molecules per activated ion channel (14), a maximal rate of ≈490 sec^{-1} was obtained. For activation by AcCho, a slightly higher value (≈670 sec^{-1}) was estimated. By using the maximal flux rate obtained for CarbCho, a transport number of 2×10^6 (ions per reconstituted channel) per second was calculated (see *Appendix*).

DISCUSSION

Recent reports have described reconstitution of membranes derived from crude (28, 29) and purified (15–18) receptor preparations that translocated cations ($^{22}Na^+$) in response to an agonist. In all cases, transport was measured on the time scale of seconds and the response was an integrated signal giving only the amplitude of the ion translocation event. We have measured the kinetics of ion transport across a reconstituted AcChoR-containing membrane by stopped-flow spectroscopy, using a recently developed technique (14) that permits continuous measurement of ion flux.

In a reproducible manner, a rapid acceleration of fluores-

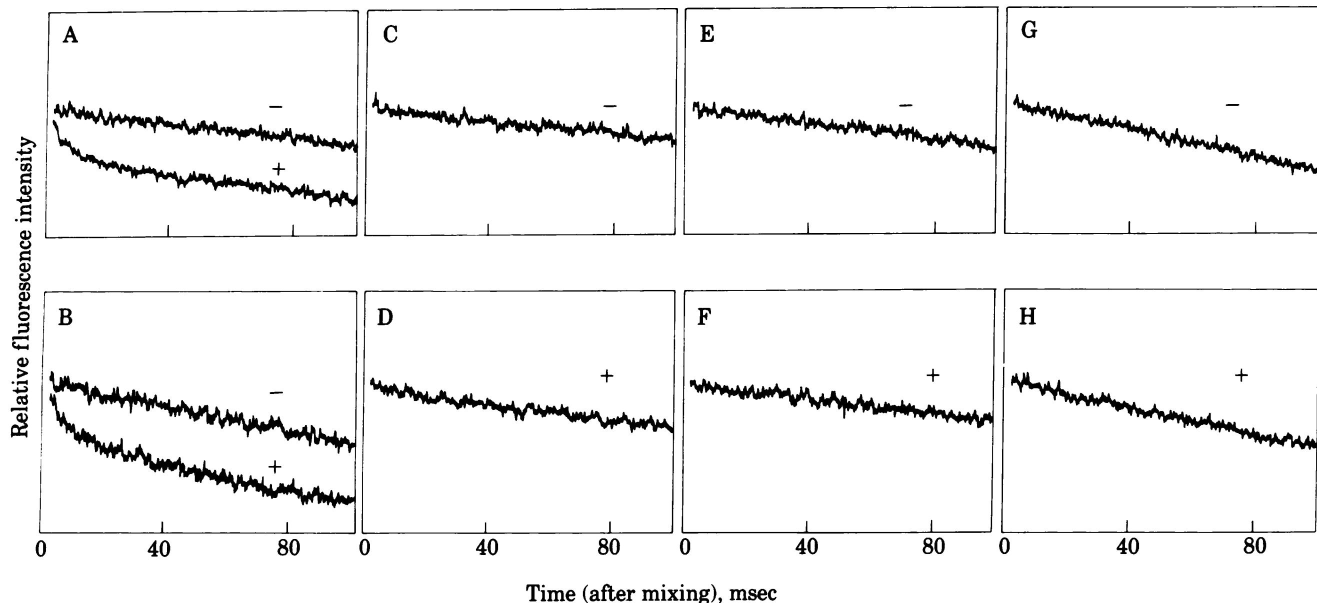

FIG. 2. Kinetics of CarbCho-induced influx of Tl^+ across reconstituted AcChoR membranes. All fluorescence traces shown were obtained by computer averaging of five or six data sets. (*A* and *B*) Reconstituted AcChoR vesicles loaded with ANTS (syringe 1) were rapidly mixed at time = 0 in a stopped-flow instrument with $TlNO_3$/buffer (syringe 2) containing no CarbCho (−) (upper trace) or with 2 mM CarbCho (+) (lower trace); fluorescence was recorded over period of 100 msec. Fluorescence traces shown were obtained from flux experiments with two different reconstituted preparations. (*C* and *D*) Blockage by HTX. Reconstituted AcChoR vesicles that contained ANTS and had been preincubated with 10 μM HTX for 5 min at 25°C (syringe 1) were rapidly mixed with 10 μM HTX in $TlNO_3$/buffer containing no (*C*) or 2 mM (*D*) CarbCho (syringe 2). (*E* and *F*) Blockage by receptor desensitization. Reconstituted AcChoR vesicles that contained ANTS and had been preincubated with 100 μM CarbCho for 30 min at 25°C (syringe 1) were rapidly mixed with $TlNO_3$/buffer containing no (*E*) or 2 mM (*F*) CarbCho (syringe 2). (*G* and *H*) Influx into liposomes lacking AcChoR. Liposomes were prepared in the same manner as reconstituted AcChoR membranes except that AcChoR was not added; they were similarly loaded with ANTS. Liposomes containing ANTS (syringe 1) were rapidly mixed with $TlNO_3$/buffer containing no (*G*) or 2 mM (*H*) CarbCho (syringe 2).

cence decay was obtained by rapidly mixing reconstituted AcChoR-containing membranes with CarbCho or AcCho; six different preparations all responded in the manner shown in Fig. 2 *A* and *B*. Specific pharmacological tests on the reconstituted AcChoR containing vesicles and similar flux experiments carried out by using liposomes lacking AcChoR suggest that the observed rapid fluorescence decay indeed represented AcChoR-mediated transport of Tl^+ into the vesicles. The rate of fluorescence decay accelerated from a half-time of 900 msec in the absence of CarbCho to 5 msec when 1 mM CarbCho was added. The apparent rate constant for agonist-independent fluorescence decay (leakage) for reconstituted membranes is about 10-fold higher than that for native membranes (decay half-time of ≈10 sec; ref. 14). This difference could be accounted for if the apparent rate constant for Tl^+ leakage is proportional to the ratio of the surface area to the interior volume of the membrane vesicles; the average diameter of the reconstituted AcChoR vesicles is approximately an order of magnitude smaller than that of native membranes.

Two important questions regarding the properties of the AcChoR are answered by these experiments. The first is whether the purified AcChoR molecule can be reconstituted after dissolution in detergent to exhibit the full functional properties of the physiological receptor in terms of its ability to transport cations. From the analysis of the ion-flux kinetics (see Fig. 3), we estimate (see *Appendix*) that at least 2×10^6 cations are transported through each reconstituted AcChoR channel per second. This transport rate compares favorably with that estimated for purified native membranes (≈10^6 sec^{-1}; ref. 14). Because the total cation concentrations used in the two preparations were different (four times higher in the reconstituted system), comparison should be made after normalizing the cation concentrations. Assuming that the number of ions transported through each AcChoR channel per second is directly proportional to the bulk cation concentration, the transport rate for the reconstituted AcChoR would be lower than, but within a factor of 2 of, that for native membranes. These numbers are within an order of magnitude of that determined for an *in vivo* AcChoR (≈10^7 sec^{-1}; refs. 25–27). The difference may be ascribed to receptor inactivation, membrane potential, and the limited probability for channel opening on agonist binding (30).

The second important implication of the results is that the AcChoR molecule constitutes the whole assembly necessary for physiological function: A pentameric complex composed of two identical subunits of 40,000 daltons and three homologous subunits (1) of 50,000, 60,000, and 65,000 daltons contains both the elements necessary for agonist or other ligand binding and the elements necessary for ion transport.

APPENDIX

The number of ions transported per second by each activated AcChoR in a reconstituted membrane vesicle can be estimated by dividing the apparent ion transport rate across a single vesicle by the average number of AcChoR molecules per vesicle.

The apparent ion transport rate across a single vesicle is calculated as follows: The maximal rate constant, obtained at saturating concentrations of CarbCho, is 487 sec^{-1} for Tl^+ transport and, under our experimental conditions (Fig. 3), the sum of the cation concentrations on either side of the vesicle membranes is 0.420 M. The rate of bidirectional cation flux is therefore 487 $sec^{-1} \times 0.42$ M = 204.54 M sec^{-1}. The interior volume of vesicles having an average diameter of 500 Å (or an inner diameter of 400 Å after subtracting membrane thickness) is 3.27

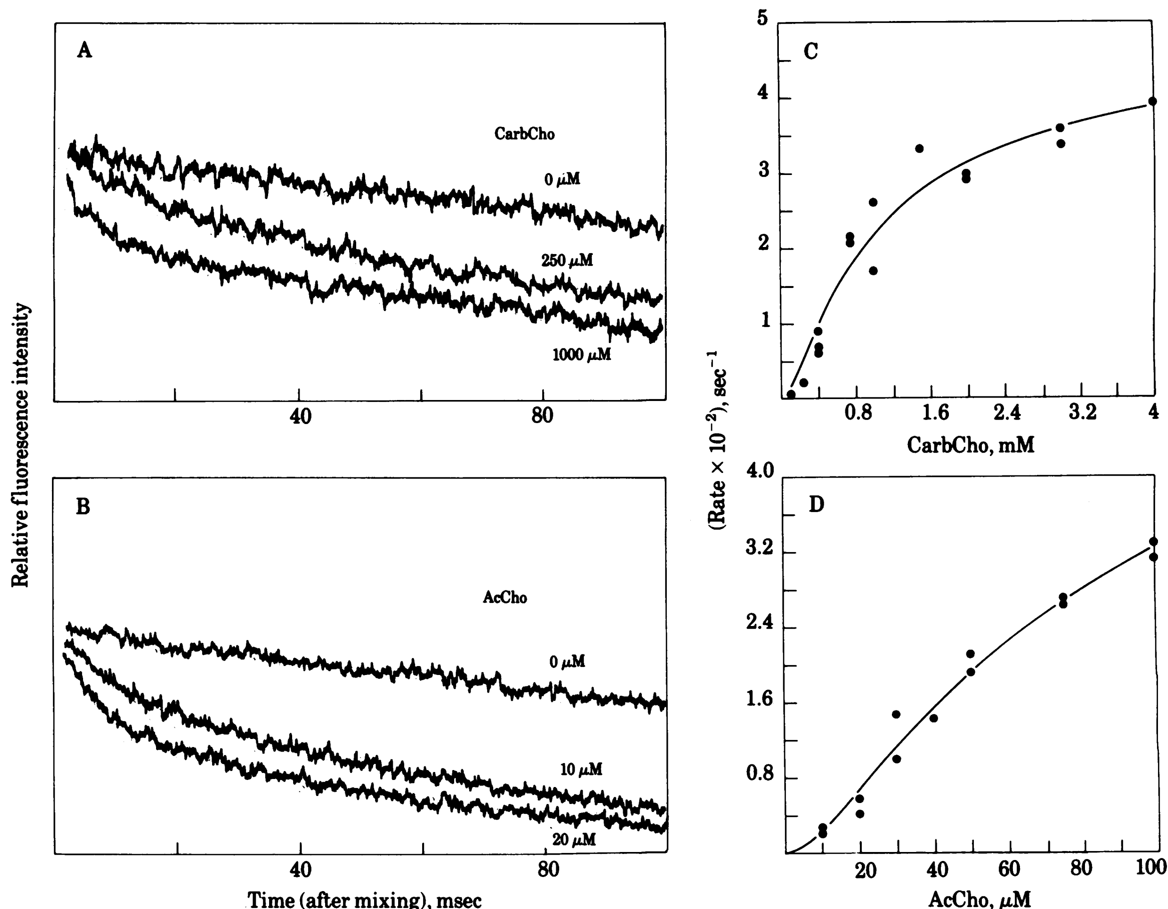

FIG. 3. Dependence of Tl^+ flux rate on agonist concentration. (*A* and *B*) Kinetics of Tl^+ influx across reconstituted AcChoR-containing membranes activated by 0, 250, and 1000 μM CarbCho (*A*) and by 0, 10, and 20 μM AcCho (*B*). (*C* and *D*) Apparent flux rates as a function of concentration of CarbCho (*C*) or AcCho (*D*). Kinetic traces obtained from computer averaging of five or six fluorescence traces for each agonist concentration were computer fitted to equation 1 of ref. 14, which describes the fluorescence decay as a function of time. The K_{app} determined was plotted against the corresponding agonist concentration. Flux experiments using final agonist concentrations >750 μM CarbCho or 30 μM AcCho were performed in the presence of 1 μM HTX, and the K_{app} obtained was normalized to the expected control value (without HTX) by a factor of 2 (empirical factors averaged from three different reconstituted preparations were 1.81 for CarbCho and 1.90 for AcCho). Plotted rates were fitted to a binding isotherm that assumed association of two agonist molecules with each activated ion channel; i.e., $K_{app} = K_{max}/(1 + K_d/\text{vol})^2$. For activation by CarbCho (*C*), $K_{max} \simeq 490\ \text{sec}^{-1}$ and $K_d \simeq 500$ μM. For AcCho (*D*), $K_{max} \simeq 670\ \text{sec}^{-1}$ and $K_d \simeq 44$ μM.

$\times\ 10^{-20}$ liter. Thus, $\approx 4 \times 10^6$ ions are transported into each vesicle per second.

The average number of AcChoR oligomers in each vesicle is estimated as follows: The number of phospholipid molecules (surface area, 55 Å^2 per molecule) that constitute a vesicle having a diameter 500 Å and a bilayer thickness of 50 Å is 2.33 $\times 10^4$. From the concentrations of α-BuTx binding sites (0.215 μM) and of phospholipids (3 mM), it is estimated that, on the average, 1.67 α-BuTx sites are associated with each vesicle. By taking into account the distribution of α-BuTx binding sites occurring as monomer and dimer (0.52:0.48, ref. 20) and assuming that two α-BuTx binding sites are associated with a receptor monomer in the reconstituted preparation, the number of receptor oligomers per vesicle is 0.64. As ≈90% of the reconstituted AcChoR is oriented with the α-BuTx binding sites on the outer surface of the vesicles (20), the average number of receptor oligomers having the correct orientation for agonist binding is 0.58. If the AcChoR oligomers are distributed among the vesicles according to a Poisson dsitribution ($n = 0.58$), the average number of AcChoR oligomers present in those vesicles containing at least one oligomer would be 1.32 per vesicle. If both ion channels associated with an AcChoR dimer are active in cation transport, there will be 1.96 channels per vesicle. Dividing the total ion transport rate across each vesicle (4×10^6 ions per sec) by 1.96 AcChoR channels per vesicle, we find the estimated number of ions transported through each reconstituted AcChoR at saturating agonist concentrations to be $4 \times 10^6/1.96 \simeq 2 \times 10^6$ (ions/per sec)/AcChoR.

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