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Transport of Acetylcholine in a Membrane Laminate Model of the Neuromuscular Junction

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Abstract

A laminate model of the cleft-plus-postsynaptic membrane structure of the neuromuscular junction was studied. In order to prepare a model of the postsynaptic membrane, the properties of acetylcholine (Ach) receptor-rich vesicles purified from Torpedo fish were measured. Immobilization of vesicles was demonstrated by various methods, in particular, by investigating collagen and carrageenan matrices as models of the fluid-filled fibrous matrix of the cleft. It was found that a laminated system employing a liquid membrane-containing vesicle suspension, together with a swollen collagen membrane, is an appropriate model for examining important transport/reception aspects of the cleft-plus-postsynaptic membrane structure.

Combined transport with immobilization of Ach in the liquid membrane system was elucidated and effective diffusivities in the vesicle suspension layer were calculated. Effective diffusivities of the composite system simulating the cleft and the postsynaptic membrane were evaluated as well. These data illustrate the importance of penetrant immobilization in retarding the diffusion process during neurotransmission.

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Index Entries: Transport, of acetylcholine in a membrane; acetylcholine, transport in a membrane; membrane, acetylcholine transport in.

Introduction

One of the essential biological membranous systems in animals modulates the transmission of nerve impulses at synapses or neuroeffector junctions (*1, 2*). Recently, a comprehensive review of the response to acetylcholine in the neurotransmission process has been provided by Lester (*3*). Lester elucidates graphically that the fusion of vesicles containing acetylcholine with the presynaptic membrane results in acetylcholine release into the fluid-filled cleft between the terminal and the muscle cell. Then, the molecules of acetylcholine penetrate the synaptic cleft and bind to receptors embedded in the muscle-cell membrane, allowing both sodium and potassium ions to counterflow through the membrane.

Our ultimate goal is to characterize experimentally the basic phenomena of the transport process across the synaptic cleft–postsynaptic membrane combination in which the matrix is comprised of collagen and mucopolysaccharide fibers containing immobilized acetylcholinesterase. From previous work, the sharpening effect of enzyme reaction on local-penetrant concentration gradients had already been isolated. Therefore, we elected to omit the enzyme from our model systems in this work so as to be able to focus more directly on the role of the receptor alone. In a recent paper (*9*), Ach iodide (AchI) and collagen were selected as models of the penetrant and the fluid-filled collagenous matrix of the synaptic cleft, respectively. Experimental data on transport and sorption under the conditions of various AchI concentrations were measured. In addition, accumulations of AchI at low bulk concentrations in the vesicles that comprise Ach receptor-rich membrane purified from Torpedo fish were measured, since the electroorgans of Torpedo fish are well-known as a source of the Ach receptor protein for biochemical experiments (*4–6*).

In this paper of our series, in order to demonstrate the transport characteristics of model membranes for the synaptic cleft and Ach receptor toward neurotransmitters such as Ach, various immobilized preparations of vesicles using collagen, carrageenan, and porous membranes are studied. Transport of Ach through synaptic model membranes is investigated, elucidating the performance of Ach and $^{22}Na^{+}$ uptakes of vesicles purified from Torpedo fish. These data are analyzed and discussed with reference to the behavior of actual nerve–muscle membrane systems.

Experimental

Materials

Microfibrillar collagen hydrochloride (collagen) and carrageenan were supplied by FMC, Inc. (Princeton, NJ). Acetylcholine iodide was purchased from Kodak Co.,

Ltd. (Rochester, NY) and radioactive AchI [^{14}C(U)] (NEC-350, 4.8 m Ci/mmol, 47.1 mg) and radioactive $^{22}Na^+$ (0.2 m Ci/mL), from New England Nuclear Co. (Boston, Mass.). Liquid scintillation cocktail (Ready-Solv™HP) was purchased from Beckman Instruments, Inc. (Fullerton, CA). Torpedo California electroplax organs (Pacific Biomarine Co., CA) obtained from freshly killed animals were frozen in liquid nitrogen and stored at −90°C until used. Microporous cellulose membranes (Grade GA-1, mean pore size 5 μM) were purchased from Gelman Sciences, Inc. (Michigan, IL) and dialysis membranes (molecular weight cutoff; 12,000–14,000) from Spectrum Medical Industries, Inc. (Los Angeles, CA). A Beckman liquid scintillation counter (Model LS-133) was used for radioactive AchI measurements. Glass distilled water was used in all procedures. Other reagents were commercially available, i.e., analytical reagents or laboratory-grade chemicals.

Purification and Reconstitution of Vesicles from Torpedo Fish

Standard procedures used to purify vesicles, including the functionally active acetylcholine receptor from the electroorgan of Torpedo fish, are described in detail elsewhere (*4*). The method used in these experiments after purification of vesicles is outlined as follows: vesicles were resuspended in flux buffer or reconstitution buffer (2% Na cholate, Sigma; 25 mg/mL of soybean L-α-phosphatidylcholine, Sigma; 100 m*M* phosphate buffer, pH 7.5; 10 m*M* NaN_3) at concentrations from 0.02 to 1.0 g vesicles/mL. Vesicle suspensions were used directly for AchI uptake studies and were used as well in diffusion studies with liquid membrane models of the postsynaptic membrane.

AchI-(^{14}C) and Carbamylcholine-Induced $^{22}Na^+$ Uptake of Reconstituted Vesicles

AchI-(^{14}C) uptake was conducted at room temperature. In microfuge tubes, 430 μL of AchI solution (10^{-4} mol/L), 20 μL of AchI-(^{14}C) solution (20,000 cpm/0.1 mL), and 45 μL of flux buffer (10 m*M* Na phosphate, pH 7.8; 400 m*M* NaCl, 5 m*M* EDTA, 0.02% NaN_3, 5 m*M* iodoacetamide) were mixed. The assay was initiated by pipeting in 5 μL of vesicles solution (ca., 0.05 g/mL) in flux buffer with an Eppendorf pipeter. After mixing by five up-and-down strokes of the pipeter, the mixture was transferred to a vial in which 0.5 g of wet Dowex 50W-X8-100 (Sigma) treated with Trizma base (Sigma) was added to ionically adsorb excess AchI in the mixture. And then, sucrose solution (3 mL of 175 m*M*) was added, and the mixture was gently shaken for 1 min. One-half milliliter of this mixture was pipeted into a liquid scintillation cocktail (10 mL), and AchI concentrations in vesicles were measured by the method of radioassay.

The $^{22}Na^+$ uptake of reconstituted vesicles was carried out by almost the same method as above. The detailed method is described in a previous paper (*5*).

Preparation of the Collagen Membrane

A suspension (30 g) containing 0.5% (w/w) of collagen was ground for four periods of 30 s each at high speed in a commercial Waring blender and then deaerated

for 30 min by a vacuum pump. The dispersion (i.e., 5 g) obtained was cast in Petri dishes (60 mm diam.) made of polystyrene. After evaporation for a few days, dry collagen membrane was aged in an incubator at 55°C for 10 d. Collagen membranes obtained had a degree of swelling of close to 3.0.

Several Vesicle Immobilization Methods for Postsynaptic Model Membranes

Collagen-Vesicle Membranes Preparation of collagen-immobilized vesicles is described in the outline as follows: Suspended collagen (0.5 w/w%) was dialyzed against distilled water to reach pH of 6.5 to 7.0 for a few days. After dialysis, vesicles (0.1–1.0 g) and dialyzed collagen (1.0 g) were mixed by a spatula at 4°C. The mixture was poured into Petri dishes (18 mm diameter) and dried at 4°C for a week.

Carrageenan-Vesicle Membranes This method is also generally useful for immobilization of enzymes and can be carried out under "wet" conditions. Carrageenan solutions (1–4 w/w%) in flux buffer were prepared at 60°C to dissolve carrageenan completely and were cooled down to 30°C just before gelation of carrageenan solution. Pellets of vesicles (0.1–1.0 g) were suspended into cooled carrageenan solutions (1.0–5.0 g). The mixture was immediately cast into the space (1–3 mm) between two glass plates at 30°C to avoid denaturing, because this temperature was relatively high for vesicles of Torpedo fish to be kept stable. Then, this mixture was kept in a refrigerator at 4°C for a few hours and immersed into 2*M* potassium phosphate flux buffer instead of sodium phosphate to coagulate the carrageenan.

Liquid Membranes Liquid membranes consisting of vesicle suspensions in porous membranes were applied as models of the postsynaptic membranes in neurotransmission. Pellets of vesicles (1.0 g) were suspended into flux buffer (4.0 mL) and made up to 5 mL as basic vesicle suspensions. Then, volume fraction of vesicles in the suspension was 0.200 in all cases. A microporous cellulose membrane was dipped in the vesicles suspension to be impregnated with the Ach receptor-rich vesicles. Both sides of the wet microporous membrane containing the vesicle suspension were attached to collagen membranes previously swollen in flux buffer. Figure 1 shows both a schematic diagram of the membrane system and a graphical depiction of the postsynaptic membrane (*10*). These membrane laminates were inserted into diffusion cells for subsequent studies.

Time-Lag Experiments

Previous time-lag experiments (*7*) were scaled down because of the relatively small quantity of available vesicles and the use of radioactive AchI in this study. The continuous flow system is shown in Fig. 2. A flow rate (q, 1.55 mL/min) of recycled "hot" AchI solution and Na-phosphate buffer (0.05*M*, pH 7.0) was pumped into cells by a Masterflex pump (Model 7014, Cole-Parmer Inst. Co., Chicago, IL). Hot AchI solutions were prepared to permit addition of 0.5 mL of radioactive AchI solution (20,000 cpm/0.1 mL) into 10 mL of cold AchI solution at a given concentration. The effective membrane area (A) was 0.950 cm^2 (11 mm

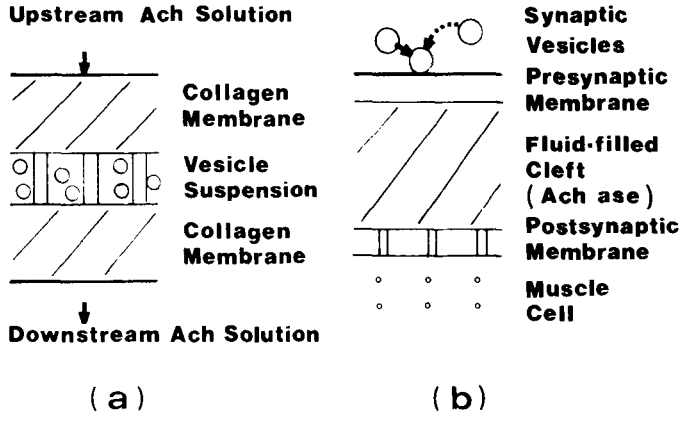

Fig. 1. Schematic diagram of the liquid membrane system (a) and postsynaptic membrane (b).

diameter). Cell volume (V) of the diffusion-cell (downstream side) was 0.250 mL. In order to avoid establishing a concentration-polarized unstirred layer at the surface of the membrane, magnetic stirrers in both cells were used. The total apparatus was kept in cold water with crushed ice (3°C). The downstream AchI solutions were collected for 1 min to measure the concentration of AchI by radioassay. The total amount (Q_t) of penetrant (AchI) that permeates through the membrane in time (t) can be calculated as follows:

$$Q_t = \int_0^t JA\, dt = \int_0^t (V dx/dt|_t + qx) dt \tag{1}$$

where J, flux of AchI, is the number of moles of AchI transported per unit area and time, and x is the downstream concentration of AchI. Q_t calculated by Eq. (1) against time displays a nonlinear transition region asymptotically tending to a steady state, represented by a straight line. It intercepts the time axis at a point θ, called the time-lag. The diffusivity (D) of the penetrant can be determined as follows:

$$D = L^2/6\theta$$

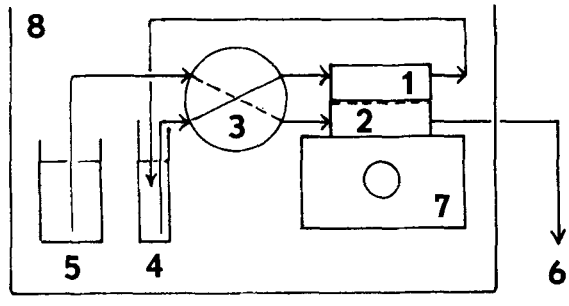

Fig. 2. Apparatus for measuring diffusivities of liquid membrane systems: (1) liquid membrane system (11 mm diameter); (2) diffusion cell; (3) Masterflex pump (1.55 mL/min); (4) recycled radioactive Ach solution (10 mL) (upstream); (5) flux buffer (pH 7.5); (6) microfuges (1.5 mL) for sampling; (7) stirrer; and (8) water bath (3°C).

where L is wet membrane thickness or total thickness of the liquid membrane laminate.

Results

Properties of Vesicles Using Acetylcholine and Carbamylcholine Induced $^{22}Na^+$ Uptake

The pH profiles of vesicles suspensions and reconstituted vesicles were examined by acetylcholine (Ach) uptake and carbamylcholine (Cch) induced $^{22}Na^+$ uptake, respectively, and the results obtained are shown in Fig. 3. Optimum pH range of Ach uptake was from 7.5 to 7.8, broader than that of Cch induced $^{22}Na^+$ uptakes of reconstituted vesicles. Figure 4 shows the thermostability of the vesicle suspension (0.050 g/mL) for 15 min at a given temperature. Ach uptake of vesicles decreased remarkably at more than 30°C and complete inactivity of vesicles was observed at 60°C. Meanwhile, Ach uptake of vesicles retained 80% of its initial activity under the conditions of thermostability at 25°C for 120 min. Inactive vesicles which were kept for 15 min as shown in Fig. 4 were no longer regenerated by reincubating at 4°C for a few minutes. Figure 5 shows lifetime of vesicles and reconstituted vesicles at 4°C. Ach uptakes of vesicles were constant within 2 months in the case of both vesicles in flux buffer and those in reconstitution buffer. Cch (10^{-3} mol/L) induced $^{22}Na^+$ uptakes abruptly decreased within a few weeks and no activity of reconstituted vesicles remained after a month. Vesicles in flux buffer without reconstitution by soybean lipids indicated no activity of Cch induced $^{22}Na^+$ uptakes.

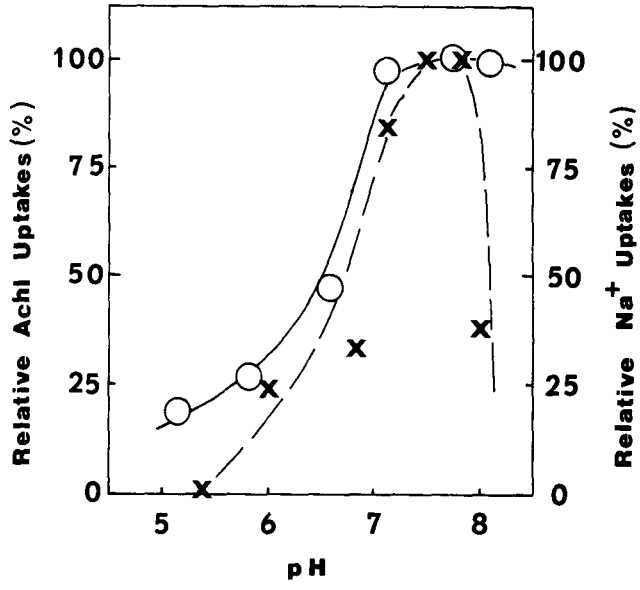

Fig. 3. pH Profiles of vesicles for Ach uptake and Cch-induced $^{22}Na^+$ uptake, ○ Ach; x Na.

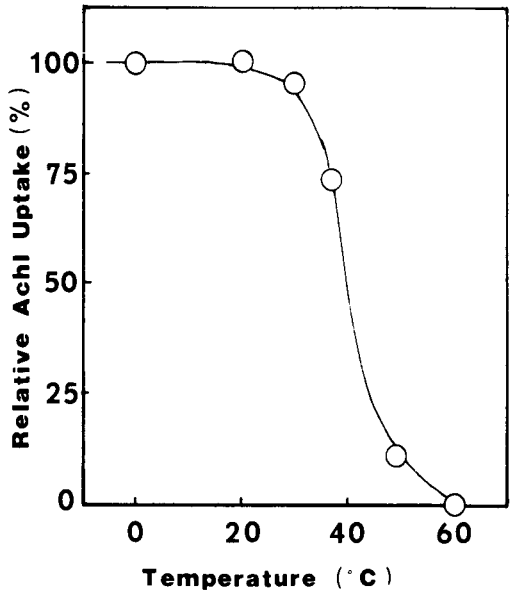

Fig. 4. Thermostability of vesicles in flux buffer after 15 min exposure.

Artificial Model of the Postsynaptic Membrane Using Vesicles

Different trials to immobilize vesicles were carried out to obtain model postsynaptic membranes. The results for various immobilization methods of vesicles are summarized in Table 1. Collagen and carrageenan were individually selected and examined as host matrixes for immobilized vesicles, since Ach receptors are embedded and bound in vivo on the outer edge of a collagenous matrix containing

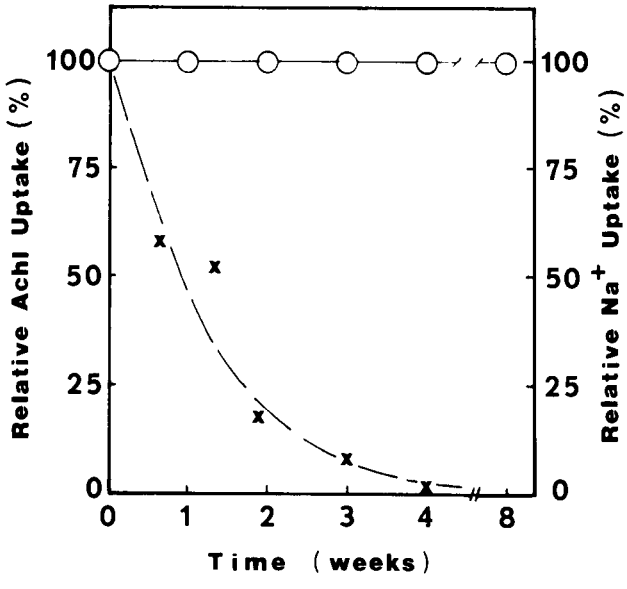

Fig. 5. Life time of vesicles for Ach uptake and Cch-induced $^{22}Na^{+}$ uptake at 4°C, ○ Ach; x Na.

TABLE 1
Vesicle Immobilization

Materials	Activity vesicles after immobilization	Methods
Collagen	No	Entrapment
Carrageenan	No	Entrapment
LMS[a]	Yes	Impregnation of micropores

[a]Liquid Membrane System.

polysaccharide, as shown by Lester (*10*). Pelleted vesicles were immobilized onto collagen by the method of a previous paper (*5*). However, the low pH (3.0–4.0) of the suspended collagen to which the pelleted vesicles were added, apparently resulted in critical denaturing of vesicles. Therefore, suspended collagen was dialyzed against water or flux buffer (pH 7.8). Stable dialyzed collagen suspension reached pH 6.5 in a few days. The pH stability of vesicles in the collagen suspension under the conditions of pH 6.5 for 24 h gave 20% of initial activity of Ach uptake. No active collagen–vesicle membranes were obtained by the method using dialyzed collagen described in the experimental section, though the activity of vesicles in the collagen suspension (pH 6.5) was still retained. Further dialysis raised the pH of the collagen suspension to 7.0. However, collagen was coagulated and, consequently, no mechanically strong collagen–vesicle membrane was obtained. Dehydration during the preparation of collagen membranes might have resulted in the denaturing of the vesicles.

In the next step, carrageenan was used for immobilization of vesicles. This method has advantages in that "wet" preparation can be used without the need to make a dehydrated membrane. Carrageenan membrane is basically formed in a sol–gel transformation that depends on carrageenan concentration, temperature, and potassium ion concentration. Balanced conditions, with carrageenan concentrations of 2% (just high enough for the vesicle–membranes to be strong), and a preparation temperature of 30°C (in which the denaturation of vesicles could be partially avoided) were experimentally selected. The effect of potassium ions (using potassium phosphate) on activity of Ach uptake of vesicles was examined. This effect is shown in Fig. 6. Activity of vesicles still remained, even in the high ionic strength environment. Therefore, the mixture of vesicles and carrageenan in flux buffer was immersed in a 2*M* potassium phosphate (flux buffer) instead of sodium phosphate to coagulate the carrageenan macromolecules. Although vesicles immobilized in carrageenan were completely inactive, vesicles suspended in a flux buffer including low content of carrageenan were nevertheless active. A small amount of calcium is an essential requirement for carrageenan gel rigidity. Cation contents of carrageenan are characterized as follows: Na (6.0%), K (0.98%), Ca (0.23%), and Mg (0.22%). It is thought that strong interactions between active sites of vesicles and cations, especially calcium, cause site inhibition or denaturation of vesicles.

Vesicle suspensions were then impregnated in preformed microporous membranes of collagen or cellulose (dialysis membrane). This liquid membrane type of

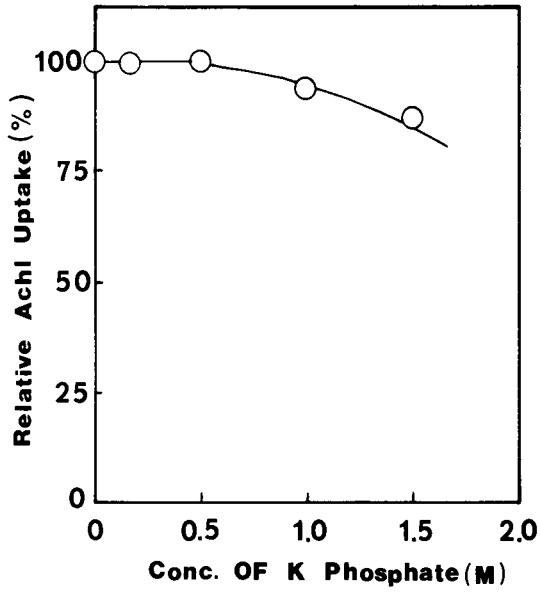

Fig. 6. Effects of ionic strength of potassium on Ach uptake of vesicles. These experiments were carried out at a variety of concentrations of potassium phosphate instead of 10 m*M* Na phosphate in flux buffer.

system avoided the denaturing steps alluded to earlier, while retaining the desired structural features we were seeking. Further experiments were carried out by the use of the liquid membrane system, which was composed of vesicle suspensions impregnated into microporous cellulose and laminated to collagen membranes.

In order to estimate the diffusion rate of neurotransmitter in the postsynaptic membrane, transport of Ach through the liquid membrane model was measured. The effects of Ach concentrations in upstream solutions on time-lag in the transport process, under the conditions of constant volume fractions of vesicle suspensions (0.05), collagen membrane thickness (ca. 150 μm), and temperature (3.0°C) are shown in Fig. 7. The time-lags asymptotically decreased with increasing upstream Ach concentrations, approaching 24 min when using vesicles and 20 min for the vesicle-free control. Effects of volume fractions of vesicles in microporous membranes on time-lags of the liquid membranes are also shown in Fig. 8. These experiments were carried out under standard conditions: 10^{-3} mol/L Ach concentration of upstream solutions, 3.0°C temperature, ca. 150 μm collagen membrane thickness, 100 μm microporous cellulose membrane (5 μm pore size), and a range of vesicle volume fractions from 0.02 to 0.20. Time-lags gradually increased with increasing volume fractions of vesicles in microporous membranes.

Discussion

Properties of vesicles purified from Torpedo fish were measured, in order to prepare a model of the postsynaptic membrane that comprised, as the cleft, the collagenous matrix and the Ach receptor impregnated as a liquid membrane into a microporous cellulose host membrane. Under the conditions for active vesicles de-

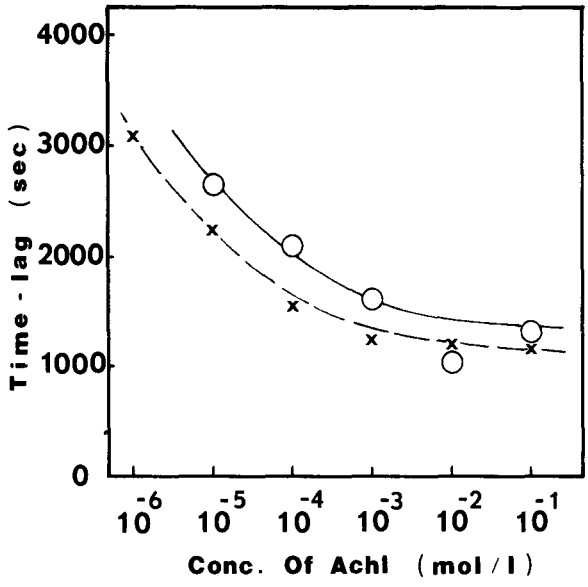

Fig. 7. Effects of Ach concentrations of upstream solutions on time-lags in the liquid membrane system; volume fraction, 0.05 (○) vesicle suspensions in the liquid layer, (x) flux buffer in the liquid layer.

rivable from Figs. 3–5, vesicles were immobilized as liquid membranes. These membranes were laminated to collagen membranes on one or both sides. As already mentioned, these were joined together to model the cleft and postsynaptic membrane combination. In this manner, the liquid membrane system was made available for investigation of transport of Ach. The time lags (θ_{121}) of the liquid

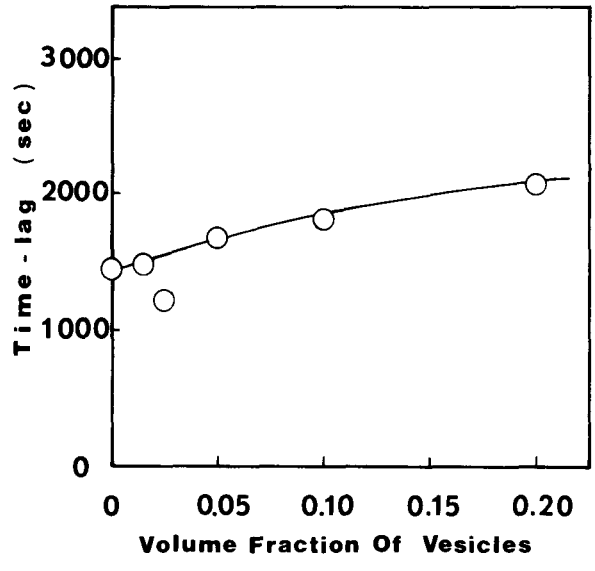

Fig. 8. Effects of volume fractions of vesicles in microporous membranes on time-lags in the liquid membrane system; Ach concentrations upstream, $10^{-}3$ mol/L.

membrane systems were measured under conditions of various Ach concentrations in upstream Ach solutions and various volume fractions of vesicles in microporous membranes.

In general, for a three-layer composite (1) (2) (1), it is shown that (*8*)

$$\theta_{121} = \frac{\frac{l_1^2}{D_1}\left(\frac{4l_1}{3D_1K_1} + \frac{l_2}{D_2K_2}\right) + \frac{l_2^2}{D_2}\left(\frac{l_1}{D_1K_1} + \frac{l_2}{6D_2K_2}\right) + \frac{l_2l_1^2K_2}{(D_1K_1)^2}}{\frac{2l_1}{D_1K_1} + \frac{l_2}{D_2K_2}} \tag{3}$$

where θ_{121} denotes the time-lag of the three layer composite, and l_1, D_1, and K_1 are the thickness, diffusivity, and distribution coefficient of the first layer, respectively. Subscripts of 1 and 2 denote the kind of membrane, that is, whether collagen layer or vesicle suspension layer. For this we had already determined that $D_1 = 2 \times 10^{-6}$ cm^2/s; K_1 is likewise obtained from previous work (*9*).

For the distribution coefficient of the vesicle suspension layer, K_2, we have

$$K_2 = [(1 - \epsilon) + \epsilon K_v]/[(1 - \epsilon) + \epsilon] = 1 + \epsilon(K_v - 1) \tag{4}$$

where K_v is the distribution coefficient of pelleted vesicles purified from Torpedo fish (*9*) and ϵ is the volume fraction of pelleted vesicles in the vesicle suspension layer. Experimental data of l_1, l_2, and ϵ were obtained from this work. Therefore, values of D_2 can be calculated from Eq. (3); they are summarized in Table 2. The diffusivities of vesicle suspension layers decreased with decreasing Ach concentrations of upstream Ach solutions and increasing volume fractions of vesicles.

Furthermore, it is thought that Ach in neurotransmission passes through the cleft (collagenous membrane) and Ach receptor-rich membrane, being regulated by the gradient of Ach concentrations between upstream and downstream solutions. The neurotransmission system for the cleft plus postsynaptic membrane combination is considered to be a two-layer composite of (1) and (2). Thus,

$$l_{12}/D_{12}K_{12} = l_1/D_1K_1 + l_2/D_2K_2 \tag{5}$$

where

$$K_{12} = (l_1K_1 + l_2K_2)/l_{12}$$

Therefore, D_1/D_{12} can be written as

$$\frac{D_1}{D_{12}} = \frac{l_1}{l_{12}^2}\left(l_1 + l_2\frac{K_2}{K_1}\right) + \frac{l_2}{l_{12}^2}\frac{D_1}{D_2}\left(l_1\frac{K_1}{K_2} + l_2\right) \tag{6}$$

Taking $K_2 = K_v$ at $\epsilon = 1.0$, and D_1 and K_1, 10^{-6} cm^2/s and 10, respectively, to allow for penetrant immobilization by the polysaccharide moieties, D_1/D_{12} can be calculated, for the case where l_1 and l_2 are taken as 900 and 100 Å, respectively. The calculated ratio is close to fifteen, so that D_{12} is estimated at about 6×10^{-8} cm^2/s.

These results clearly show the retardation of the diffusion process ($D_{12} < D_1$) that occurs because a fraction of the penetrant population is immobilized (K_2) and

TABLE 2
Trend of Diffusivities of Ach in Vesicle Suspension Layer: Experimental Data and Computed Results[a]

No.	Ach upstream conc., mol/L	Volume fraction of vesicles	Distribution coefficient			Membrane thickness, cm		Time-lag, θ_{121}, s	$D_2 \times 10^{+7}$ cm^2/s
			K_v	K_1	K_2	l_1	l_2		
1	10^{-5}	0.05	1000	12	51	0.0144	0.010	2700	1.5
2	10^{-4}	0.05	750	10	38.5	0.0150	0.010	2160	1.6
3	10^{-3}	0.05	250	8	13.5	0.0174	0.010	1690	3.2
4	10^{-2}	0.05	8	2	1.4	0.0185	0.010	1560	7.6
5	10^{-1}	0.05	1	1	1.0	0.0173	0.010	1440	5.2
6	10^{-3}	0.20	250	8	51	0.0254	0.010	1980	1.5
7	10^{-3}	0.10	250	8	26	0.0270	0.010	1830	5.8
8(3)	10^{-3}	0.05	250	8	13.5	0.0174	0.010	1690	3.2
9	10^{-3}	0.025	250	8	7.25	0.0236	0.010	1440	4.1
10	10^{-3}	0.020	250	8	6.0	0.0153	0.010	1410	5.5
11	10^{-3}	0.	1	8	1.0	0.0180	0.010	1410	50.

[a] v; vesicles; 1, collagen layer; 2, vesicle suspension layer.

unavailable for diffusion at any instant. This effect will be only partially offset by the gradient-sharpening effect of the enzyme reaction.

The rise time of the neurotransmission process is known to be about 100 μs from experimental data. Previous calculations had indicated (*10*) that the process would require only 20 μs, considering diffusion in the cleft alone. Thus, it was concluded that the diffusion process in the cleft was not controlling. However, for the composite structure of the cleft plus postsynaptic membrane, the effective diffusivity, D_{12}, is substantially reduced below D_1 because of retardation caused by receptor binding. Therefore, we conclude that the neurotransmission process of Ach can in fact be controlled by a modified diffusion process in the laminate structure.

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