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Acetylcholine Receptor-Controlled Ion Flux in Electroplax Membrane Vesicles: Identification and Characterization of Membrane Properties that Affect Ion Flux Measurements

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Summary. Several intrinsic properties of acetylcholine receptor-rich membrane vesicles prepared from *Electrophorus electricus,* which need to be considered in measurements of receptor-mediated ion flux, have been identified. One of these properties is a slow exchange of inorganic ions in the vesicles. The slow exchange of ions is not related to the receptor-mediated flux of ions and accounts for 30–35% of the efflux observed. A method to separate this process from the receptor-controlled flux has been developed. It has also been shown, using a light-scattering method, that aggregation-disaggregation of the vesicles can interfere with the efflux measurements, and a method to overcome this problem has been developed. The difference in the amplitude of effluxes induced by saturating amounts of carbamylcholine and gramicidin has been investigated and has been shown not to be due to a receptor-controlled process; therefore, the amplitude difference does not need to be considered in understanding the receptor-controlled process.

Acetylcholine receptor-rich membrane vesicles (microsacs) prepared from *Electrophorus electricus* provide an *in vitro* system in which to study interactions of the receptor with chemical mediators and the subsequent changes in the permeability of membranes to inorganic ions. There are several advantages in working with vesicles rather than with intact cells. (i) Variable and defined solution compositions inside and outside the vesicles can be used. (ii) The internal volume of the vesicles, and the number of bound charges, can easily be determined. (iii) Passive and enzyme-driven fluxes of inorganic ions do not interfere with the measurements. (iv) The number of receptors exposed to a known concentration of ligand can be determined.

The vesicles have been shown to respond to acetylcholine and other acetylcholine-receptor ligands (Kasai & Changeux, 1971*a–c*), but the population also contains vesicles that are unaffected by receptor-ligands (unspecific vesicles) in addition to vesicles that are sensitive to acetylcholine and its analogs (specific vesicles). About 85% of the total observed efflux is due to unspecific vesicles (Hess et al., 1975). The specific vesicles have been physically separated from the unspecific ones by taking advantage of their permeability differences (Hess & Andrews, 1977).

Recently it was shown (Hess, Lipkowitz & Struve, 1978) that the receptor-controlled ion flux is biphasic, an initial fast phase preceding a second slow phase. On the basis of electrophysiological experiments, Katz and Thesleff (1957) proposed the existence of a ligand-dependent equilibrium between active and inactive (desensitized) receptor states. The results of ion flux experiments could be explained on the basis that the initial fast phase was due to the active state of the receptor and the slow phase to the inactivated state (Hess et al., 1978). Although the fraction of the total efflux due to the fast process (α) increased with increasing carbamylcholine concentrations, a slow phase persisted at saturating carbamylcholine concentrations, and a maximum value for α of about 0.65 was obtained. This led to the suggestion that, even at saturating ligand concentrations, inactivation or desensitization of the receptor could occur before exchange of radioactive ions inside the vesicles with the ions in the external medium was complete. We now know that inactivation does occur, but the rate of ion movement through the (inactivated) receptor is much faster than was thought previously (Hess, Cash & Aoshima, 1979; Epstein et al., 1980). In this paper

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we show that the slow phase observed at saturating ligand concentrations, which accounts for 30–35% of the total efflux, is due to slowly exchanging ions which are not controlled by the receptor, and therefore their efflux is not related to the receptor-mediated process. Thus, our results indicate that at high ligand concentrations the ion flux in the vesicles goes to completion before the first measurement can be made with the Millipore filter assay or within 10 sec (Hess et al., 1979). The slowly exchanging ions have not been considered in efflux experiments used in qualitative investigations of receptor function (Kasai & Changeux, 1971*a–c*; Bernhardt & Neumann, 1978; Hanley, 1978; Hsiao-Ping et al., 1979).

We have also shown, using light-scattering and ion flux methods, that disaggregation of the vesicles can occur and that this can interfere with flux measurements. This also has not been considered in efflux measurements (Kasai & Changeux, 1971*a–c*; Bernhardt & Neumann, 1978; Hanley, 1978; Hsiao-Ping et al., 1979). A procedure was developed to overcome the interference produced by vesicle disaggregation and has been used to observe the time-dependent development of the slow phase described earlier.

We have also investigated the effects of a channel-forming antibiotic, gramicidin D, on the vesicles. Popot, Sugiyama, and Changeux (1976) observed an amplitude difference between the effects of saturating amounts of gramicidin and of carbamylcholine in *Torpedo* receptor-rich vesicles. Although we also observe an amplitude difference between saturating amounts of gramicidin and carbamylcholine when we use *E. electricus* receptor-rich vesicles, we show here why this amplitude difference is not related to receptor-controlled processes and why it does not need to be considered in understanding the function of such processes. In previous experiments this difference in amplitude was ascribed to inactivation (desensitization) of the receptor (Popot et al., 1976).

Materials and Methods

Electric eels were obtained from World Wide Scientific Animals, Ardsley, N.Y. The vesicles were prepared as described by Kasai and Changeux (1971*a*). Vesicles in sucrose-free solutions were prepared by pelleting the microsacs prepared in sucrose, and resuspending them in a sucrose-free buffer by the procedure of Fu et al., (1977). Carbamylcholine chloride, D-tubocurarine chloride, gramicidin D, valinomycin, and amphotericin B were purchased from Sigma Chemical Co. Stock solutions of ^{86}RbCl, and ^{22}NaCl were obtained from New England Nuclear, and Na^{36}Cl was obtained from Amersham Corporation. Tri-*n*-butyl tin chloride was the generous gift of Professor Efraim Racker. All other chemicals were reagent grade and were obtained from either Fisher Scientific or Mallinkrodt.

Stock solutions of gramicidin D (20 mg/ml), valinomycin (5.0 mM), and tri-*n*-butyl tin (10% vol/vol) were made up in absolute ethanol. Stock solutions of amphotericin B (35 mg/ml) were made up in dimethyl sulfoxide (DMSO), and stored in a brown glass container. All subsequent dilutions were made using the same solvent as was used in in the stock solutions. The final ethanol concentrations never exceeded 0.4% in the experiments. Ethanol concentrations up to 1% had no effect on the flux measurements. The final DMSO[1] concentration never exceeded 0.2% and had no effect on the flux measurements. Protein concentrations were determined using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Light-scattering measurements were made using an argon laser (Coherent Radiation Model 52) at 457.9 nm as the light source, and a monochromator (Jobin Yvon Ranamor HG2) at a 90° angle to the source.

Ion flux measurements were made essentially as reported by Hess et al. (1975). The vesicles were loaded using three different techniques (described below) with the radioactive tracer ions, $^{22}Na^+$, $^{36}Cl^-$, or $^{86}Rb^+$. The latter ion is thought to serve as an effective replacement for $^{42}K^+$ (Palfrey & Littauer, 1976) and has the advantage of being a more stable isotope. At the beginning of each experiment the loaded vesicles were diluted to a final concentration of either 160 μg or 80 μg of protein per ml with appropriate solutions containing phosphate buffer pH 7.0. These solutions included potassium eel Ringer's solution (150mM KCl, 9.4 mM NaCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1 mM phosphate, at pH 7.0) or sodium eel Ringer's solution (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1 mM phosphate, at pH 7.0) (Keynes & Martins-Ferreira, 1953). The vesicles were kept at 4 °C at all times before and during the experiment. A Millipore filtration assay was used to determine the amount of radioactive ions retained by the vesicles as a function of time. The filters (Millipore HAWP 02500, HA 0.45 μm, 25 mm) retain 95% of the vesicles, although the size of the latter is much smaller than that of the pores of the filter (Kasai & Changeux, 1971*a*). Unless otherwise specified, 80 μg of protein were used per filter for each time point.

Three different procedures for loading the vesicles with radioactive ions were used. In the first procedure (overnight loading) the vesicle preparation, about 5.0–10.0 mg of protein per ml, was incubated overnight at 4 °C in appropriate solutions containing the radioactive tracer ion. Incubations with $^{86}Rb^+$ were made at concentrations of 50–100 μCi per ml, with $^{22}Na^+$ at 25–50 μCi/ml, and with $^{36}Cl^-$ at 40–60 μCi/ml. Due to limitations in manufacturing, radioactive chlorine ($^{36}Cl^-$) is not available in concentrated stock solutions. Our supply came in 257 mM NaCl at a concentration of 110 μCi/ml. By properly adjusting the ionic composition of the vesicles before the addition of the $^{36}Cl^-$, we were able to obtain a final ionic composition in the incubation identical to sodium eel Ringer's solution. The specific activity in these incubations was 0.27 mCi/mmol.

The second procedure in which only the specific vesicles are loaded (ligand-induced loading or LIL) was to add a mixture of carbamylcholine and the radioactive tracer ion (final concentration of 100 μCi/ml) to the vesicle preparation (5.0–10.0 mg of protein per ml). This allowed the radioactive ions to move into the specific vesicles at an accelerated rate. After a specified time period, D-tubocurarine chloride (curare), an effective inhibitor of carbamylcholine, was added to stop the influx. The molar concentration of carbamylcholine never exceeded 120 times the molar concentration of curare to ensure complete inhibition. It has been shown that curare does not damage the vesicles (Cash, Hess, 1981). The incubation mixture was then diluted 50 to 100-fold before the efflux was measured.

[1] *Abbreviations used:* curare, D-tubocurarine; DMSO, dimethyl sulfoxide; LIL, ligand-induced loading; LIL-DS, ligand-induced loading in dilute solution; α, fraction of the total efflux due to the fast phase; k_{obs}, observed rate constant for the slow phase of the efflux.

A third method was developed to avoid the disaggregation phenomenon that is described later. The procedure used differs from the previous one in that the vesicles were diluted only 6- to 10-fold after loading with radioactive ions (ligand-induced loading in dilute solution or LIL-DS). A vesicle preparation was diluted to a concentration of 1.0 mg of protein per ml at least 1 hr before the start of an experiment. A solution of carbamylcholine and the radioactive tracer ion was added to a final concentration of 0.5 mM and 100 μCi/ml, respectively. After 0.30 min, the vesicles were diluted 6- to 10-fold with the appropriate buffer containing 10 mM curare.

When the overnight incubation method was used, vesicles were allowed to remain in the dilution buffer for a minimum of 120 min. By the end of this time the radioactive ions inside the unspecific vesicles had equilibrated with the external ions (Hess et al., 1975). The LIL procedure required a minimum wait of 20 min to allow the disaggregation phenomenon to go to completion. However, with the LIL-DS procedure, measurements could be made immediately after loading, since the unspecific vesicles had not been filled to any significant extent and the disaggregation phenomenon was not significant.

Results

I. Slowly-Exchanging Inorganic Ions

Efflux of $^{86}Rb^+$ from specific membrane vesicles is shown in Fig. 1. In the experiments the LIL procedure for loading the vesicles with $^{86}Rb^+$ was used under physiological conditions (potassium eel Ringer's solution inside the vesicles and sodium eel Ringer's solution on the outside). Addition of 10 mM carbamylcholine resulted in a biphasic efflux. The addition of gramicidin to the vesicles also resulted in a biphasic efflux. A slow phase persists even at saturating gramicidin concentrations, just as it persists at saturating ligand concentration (Hess et al., 1978). Control experiments were performed to be sure that the gramicidin was not breaking vesicles. Since the antibiotic is known to have no effect on anion permeability (Pressman, 1976), the vesicles were loaded overnight with $^{36}Cl^-$ and the efflux followed. Addition of saturating amounts of gramicidin had no effect on the $^{36}Cl^-$ efflux, which is also shown in Fig. 1 (○), indicating that vesicles were not broken. Saturating amounts of ligand (carbamylcholine) or saturating amounts of gramicidin both result (Fig. 1) in a slow phase that (i) accounts for approximately 35% of the total efflux and (ii) follows a single exponential decay with a half-time of approximately 3 min. Similar results were obtained when $^{22}Na^+$ was used to follow efflux. These results indicate a slow exchange of cations in the vesicles and that the process is not related to receptor-controlled fluxes.

In another experiment gramicidin was allowed to equilibrate overnight with the vesicles, but a biphasic efflux was still obtained, and the results were comparable to those obtained when gramicidin was

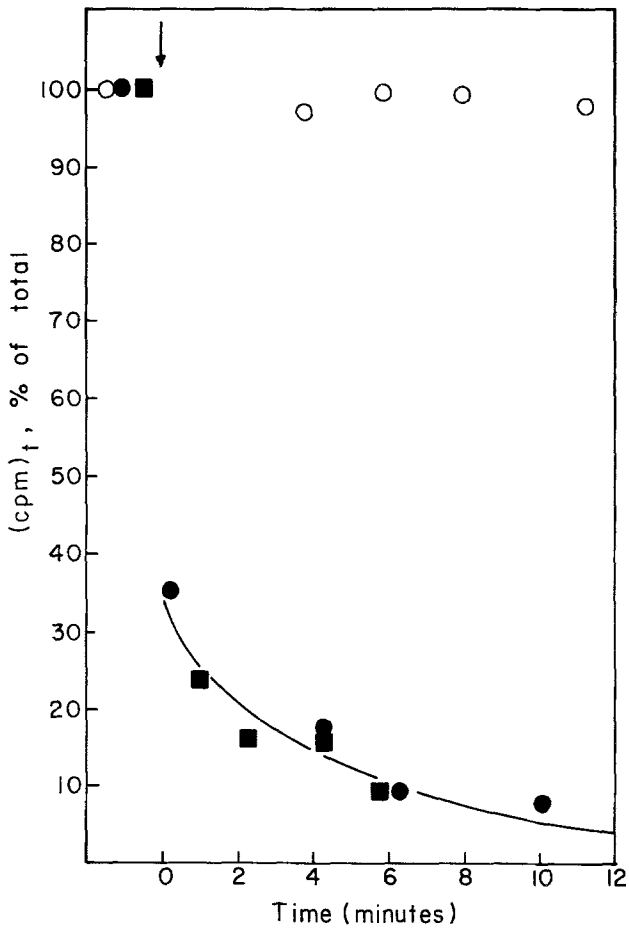

Fig. 1. $^{86}Rb^+$ efflux from specific vesicles. Vesicles in potassium Ringer's solution were loaded using LIL with 1 mM carbamylcholine and 180 μCi $^{86}Rb^+$/ml. Curare (60 μM) was added after 8 min to stop the influx. The vesicles were then diluted with sodium eel Ringer's solution to a final concentration of 160 μg of protein per ml, and 0.5 ml aliquots (80 μg protein) were taken for each point. After a 20-min period in the dilution buffer, 25 μg/ml gramicidin (●) or 10 mM carbamylcholine (■) was added. The arrow indicates the time of addition. (○) $^{36}Cl^-$ control efflux. Vesicles in sodium eel Ringer's solution were loaded overnight with $^{36}Cl^-$. The vesicles were then diluted to a final concentration of 160 μg of protein per ml. After approximately 200 min, 25 μg/ml gramicidin was added. All results are plotted as a percent of total radioactive metal ions present before addition of gramicidin or carbamylcholine

added during the efflux (*see* Table 1). We can, therefore, rule out the possibility that the slow phase of the efflux observed with gramicidin is due to a slow incorporation of the antibiotic into the vesicles.

In sodium eel Ringer's solution the concentration of cations bound inside the vesicles is 63 mM (Kim, 1979). This result suggested the hypothesis that the slowly exchanging inorganic ions were tightly bound inside the vesicles, and thus were responsible for the slow phase of the effluxes observed with saturating levels of gramicidin and carbamylcholine. We there-

Table 1. Properties of the slow exchange of inorganic ions in membrane vesicles

		Amphotericin B (1.4 μg/ml)	Amphotericin B (7 μg/ml)	Gramicidin (25 μg/ml)
$^{22}Na^+$	k_{obs}[a]	0.15 min^{-1}	0.14 min^{-1}	0.16 min^{-1}
	$(1-\alpha)$[b]	0.22	0.19	0.34
$^{36}Cl^-$	k_{obs}[a]	0.17 min^{-1}	0.16 min^{-1}	
	$(1-\alpha)$[b]	0.19	0.13	

[a] Rate coefficient for the slow exchange of ions.

[b] The fraction of ions in vesicles which exchange slowly.

Vesicles in sodium eel Ringer's solution were incubated overnight with amphotericin B (in 0.2% DMSO) or gramicidin (in 0.2% ethanol). Control experiments included incubations with 0.2% DMSO or 0.2% ethanol, and these solvents did not affect the measurements. After overnight incubation, the vesicles were diluted ~30-fold with sodium eel Ringer's solution containing the same concentration of antibiotic that was present in the incubations.

fore checked to see if the slow phase depended on salt concentration (i.e., to see if we could saturate the charges). Vesicles were incubated overnight with varying salt concentrations (50 to 500 mM KCl) and the effluxes were initiated with either carbamylcholine (1 mM) or gramicidin (25 μg/ml). For both the carbamylcholine- and gramicidin-induced effluxes, both the fraction of the total efflux due to the fast phase (α) and the observed rate of the slow efflux (k_{obs}) were independent of salt concentrations in the range tested. These results do not rule out the possibility that the molar concentration of binding sites exceeds the molar concentration of inorganic ions, 500 mM, in the experiment. In view of the estimated diameter of the vesicles and of their lipid composition (B.L. Lenchitz, R.L. Noble & G.P. Hess, *manuscript in preparation*), the existence of such sites appears unlikely. The results are also consistent with a dissociation constant of the complex which is larger than 500 mM. However, the slow exchange rates for both cations and anions are similar (*see* below), and the formation of salts involving ionizing groups of the lipids or proteins with high dissociation constants appears unlikely.

Additionally, since Ca^{2+} is known to bind many organic ligands much more tightly than Na^+ or K^+ (Sillen & Martell, 1964), we decided to check the effect of Ca^{2+} on the observed slow phase. Vesicles containing 150 mM KCl, 9.4 mM NaCl, and 1 mM phosphate were equilibrated with 0, 3, and 10 mM Ca^{2+}, and then loaded with $^{86}Rb^+$ using the LIL procedure. Effluxes induced with 20 μg gramicidin/ml were performed against buffers containing the same amount of Ca^{2+} as was in the vesicles. The results showed that Ca^{2+} did not affect the slow phase (k_{obs}) or the fast phase (α) of the $^{86}Rb^+$ effluxes.

Anions, as well as cations, are involved in the slow exchange, as shown in experiments with the channel-forming polyene antibiotic, amphotericin B. This antibiotic forms channels in membranes which conduct cations, anions, and small organic molecules with very little specificity (Pressman, 1976; Cass, Finkelstein & Krespi, 1970). NMR studies (Gent & Prestegard, 1976) have shown: (i) Amphotericin B causes vesicle breakage which is greatest when used at antibiotic/cholesterol ratios greater than 1:1. (ii) Pore formation is slow and completed only after the membranes have been exposed to the solution for about 2 days. (iii) Amphotericin B does not seem to cause structural changes in the membrane. Lipid analysis of receptor-rich membrane vesicles from the electric ray, *Torpedo californica*, has shown a protein/lipid ratio of 70:30% and a cholesterol/lipid ratio of 22:78% (Schiebler & Hucho, 1978). Assuming that the lipid content of eel membrane vesicles is approximately the same as that for *Torpedo* vesicles, we used concentrations of amphotericin B that correspond to 1:2 and 1:10 amphotericin B/cholesterol ratios (7 μg amphotericin B/ml and 1.4 μg amphotericin B/ml, respectively, for a solution containing 160 μg protein/ml).

The results (Table 1) show that amphotericin B-induced flux of both $^{22}Na^+$ and $^{36}Cl^-$ is biphasic. The observed rates for the slow exchange of Na^+ and Cl^- are similar. It should be noticed that α is large, undoubtedly due to some breakage of vesicles. The important point is that a slow phase still persists for Cl^- and Na^+.

The time-dependent development of the slow phase is depicted in Fig. 2. To avoid disaggregation problems (discussed later), the LIL-DS procedure was used for loading vesicles in potassium Ringer's solution with $^{86}Rb^+$. The vesicle mixture was then diluted with potassium Ringer's solution containing 10 mM D-tubocurarine to close the receptor channels. The solution was allowed to stand for varying periods of time (1 to 50 min) after which gramicidin (20 μg/ml) was added and the efflux measured. As the time of standing before addition of gramicidin was increased, the fraction of the efflux due to the slow phase increased (i.e., α decreased). The flux of $^{86}Rb^+$ into the vesicles is rapid in the presence of carbamylcholine, and is halted upon dilution with curare. Since the dilution was greater than sixfold, the concentration of $^{86}Rb^+$ inside the vesicles was higher than that in the dilution buffer. During the time of standing there was no flux of $^{86}Rb^+$ into the vesicles. The observed changes are, therefore, due to processes involving inorganic ions inside the vesicles. The time dependence of the slow-phase development can be fitted to a single exponential with a half-time of approxi-

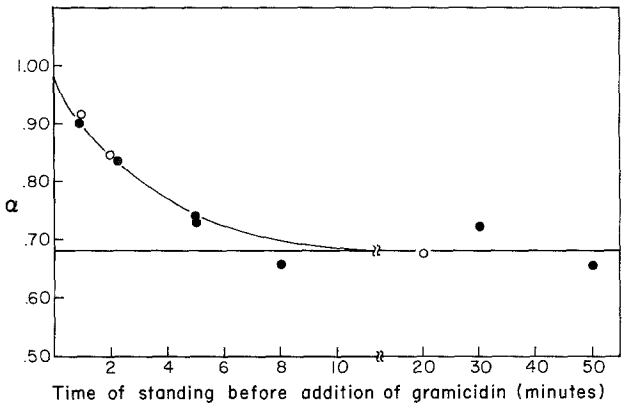

Fig. 2. Time-dependent development of the slow phase. Vesicles (1.0 mg protein/ml) in potassium Ringer's solution were loaded using LIL-DS with 0.5 mM carbamylcholine and 100 μCi $^{86}Rb^+$/ml for 0.3 min. The vesicles were then diluted to 160 μg of protein per ml with potassium Ringer's solution containing 10 mM curare. Gramicidin (20 μg/ml) was added after a specified time and an efflux curve was obtained. The experiment was repeated several times, varying the time before the addition of gramicidin. The fraction of the observed efflux due to the fast phase (α) is plotted *vs.* the time of standing before gramicidin addition. Solid symbols represent single experiments, open symbols are the average of two experiments. The exponential line shown has a half-time of approximately 3 min

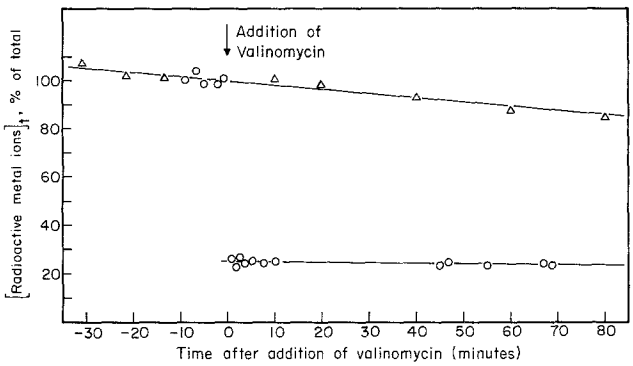

Fig. 3. Effect of valinomycin on $^{86}Rb^+$ efflux from specific vesicles (○). Vesicles (3.1 mg protein per ml) in 400 mM KCl, 100 mM sucrose, 2 mM P_i, pH 7, were loaded using the LIL procedure with 0.1 mM carbamylcholine and 160 μCi $^{86}Rb^+$/ml. After 10 min 0.1 mM curare was added and the vesicles were diluted to 160 μg of protein per ml with the same buffer. After a 20-min period in the dilution buffer, 0.5-ml samples were taken for each point. After waiting a specified period of time, valinomycin (5 μM) was added. The control experiment is the effect of 5 μM valinomycin on $^{22}Na^+$ flux from specific vesicles in 90 mM KCl, 10 mM NaCl, 0.4 M sucrose, 2 mM P_i, pH 7.0 (△). The results are plotted as a percent of total radioactivity

mately 3 min, in agreement with the efflux results (Fig. 1).

Experiments with true ionophores (not channel formers) (Pressman, 1976) produced different results. Valinomycin is one of the true ionophores which has a high specificity for K^+ and Rb^+ (Pressman, 1976). A valinomycin titration was performed and the saturation concentration was determined to be approximately 1 μM. Shown in Fig. 3 is a $^{86}Rb^+$ efflux (○) performed using 5 μM valinomycin in a Na^+-free solution. Valinomycin induced an efflux, too fast to be measured with the Millipore filter assay, which accounted for 100% of the total efflux (i.e., no slow phase was observed). Also shown in Fig. 3 is a control experiment with $^{22}Na^+$ (△) which indicates that vesicles are not broken at this concentration of valinomycin.

Another true ionophore, tri-*n*-butyl tin, known to promote Cl^- for OH^- exchange, was used (Selwyn et al., 1970). When the ionophore was used at a concentration of 1.4 μM, a $^{36}Cl^-$ efflux too fast to be measured also resulted and accounted for 100% of the total efflux. A $^{86}Rb^+$ control flux was also performed and no harmful effects of the ionophore were observed.

II. Disaggregation of vesicles upon dilution

The effect of dilution of vesicles upon efflux measured using the Millipore filter assay is illustrated in Fig. 4*a*. The results are expressed as percent of radioactivity inside the vesicles as a function of time. In the experiments shown, the vesicles were loaded for the same length of time in the presence of 100 μCi $^{22}Na^+$ per ml. The results were normalized to the $^{22}Na^+$ content of the vesicles when the efflux had ceased and equilibrium with inorganic ions in the external solution had been attained. In one experiment (○) the vesicles were loaded by the LIL procedure using 0.5 mM carbamylcholine for 20 sec; the loading was stopped with 10 mM curare. In the other experiment (□) carbamylcholine and curare were omitted during loading with $^{22}Na^+$. In both experiments the vesicles were diluted over 100-fold to a membrane protein concentration of 80 μg/ml. The initial relatively rapid phase of the efflux was seen in both experiments. This phase is not related to receptor-controlled flux since, when carbamylcholine and curare were omitted during loading, the initial apparent loss of $^{22}Na^+$ from the vesicles was still observed. This suggested that disaggregation of the vesicles was taking place upon dilution which was 100-fold or greater and that disaggregation affected the assay of radioactivity inside the vesicles. In the experiment designated by open triangles the protein concentration was only 800 μg per ml during loading with $^{22}Na^+$, and the vesicles were diluted to 80 μg membrane protein/ml before the efflux was measured. The results show that the initial apparent loss of $^{22}Na^+$ from the vesicles, which was seen in the other two experiments, was essentially

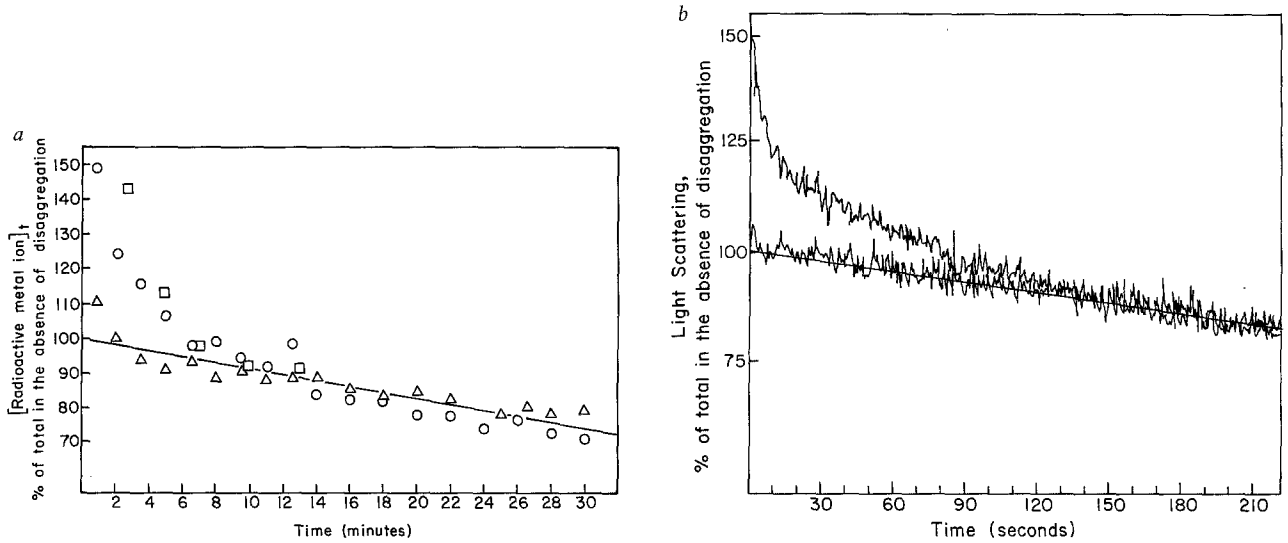

Fig. 4. Disaggregation of vesicles. (*a*): Radioactivity measurements. (○) Vesicles in sodium eel Ringer's solution (10 mg protein per ml) were loaded using the LIL procedure with 0.5 mM carbamylcholine and 100 μCi $^{22}Na^+$ per ml. After 0.3 min the vesicles were diluted 125-fold (final concentration of 80 μg of protein per ml) with sodium eel Ringer's solution containing 5 mM curare. (△) Vesicles (0.8 mg protein per ml) were loaded using LIL-DS procedure under the same conditions. The vesicles were then diluted 10-fold (same final concentration). (□) Vesicles (8 mg protein per ml) were loaded for 0.3 min with $^{86}Rb^+$ in the absence of carbamylcholine before being diluted 100-fold (same final concentration). The results are normalized to the $^{22}Na^+$ content of the vesicles when the efflux has ceased and equilibrium with inorganic ions in the external solution has been attained. (*b*): Light-scattering measurements. *Upper trace:* Vesicles (6.0 mg protein per ml) equilibrated with sodium eel Ringer's solution were diluted 150-fold to a final concentration of 40 μg protein per ml, and the light scattering measured as a function of time. *Lower trace:* Vesicles (0.6 mg protein per ml) were diluted 15-fold under the same conditions to the same final protein concentration. The results are shown as percent of light scattering, normalized to the expected light scattering in the absence of disaggregation

eliminated when the vesicles were diluted only 10-fold before the measurements were made.

Direct evidence that disaggregation occurs when the vesicles are diluted 100-fold comes from light-scattering experiments. Light scattering is known to be a useful tool in the estimation of molecular weights and can therefore be used effectively to measure disaggregation of particles (Doty & Edsall, 1951). Figure 4*b* depicts the light scattering observed upon dilution of the vesicles. The upper trace represents a 150-fold dilution and the lower one a 15-fold dilution. The final protein concentration was kept constant at 40 μg/ml. The results show that a large dilution of the vesicles resulted in an initial decrease in light scattering before a baseline level was reached, an observation consistent with vesicle disaggregation. When the vesicles were diluted only 15-fold, a change in the aggregation state of the vesicles was not detected.

The LIL-DS technique, in which dilute vesicle solutions were incubated with tracer ions and in which the vesicle solutions were diluted only 10-fold before making efflux measurements, was needed for the experiments in which we measured the appearance of the slow flux (Fig. 2). The LIL-DS technique has the advantage of (i) loading the specific vesicles completely, while not loading the vesicles which do not respond to carbamylcholine and which in subsequent efflux experiments would obscure the receptor-controlled flux (Hess et al., 1975); (ii) reducing the disaggregation phenomenon to the point where it is no longer significant; and (iii) consequently permitting measurements of the flux of ions into specific vesicles immediately after loading. This last advantage is important if one wants to avoid the effects of the slowly exchanging ions discussed in Section *I*. We have used the LIL-DS procedure to study ion movement mediated by equilibrium mixtures of active and inactive receptor forms (Epstein et al., 1980; Aoshima et al., 1980).

III. Comparison of the Amplitude of Effluxes due to Gramicidin and Carbamylcholine

When gramicidin is added to vesicles that have been incubated overnight with radioactive ions, the amplitude of the gramicidin-induced efflux is greater than the amplitude of efflux induced by saturating levels of carbamylcholine. Work in this laboratory has shown that with eel vesicles the desensitized or inactivated state of the receptor still allows ions to flow through receptor-formed channels with a half equilibration time, $t_{\frac{1}{2}}$, of less than 30 sec (Hess et al., 1979; Epstein et al., 1980). However, as shown in

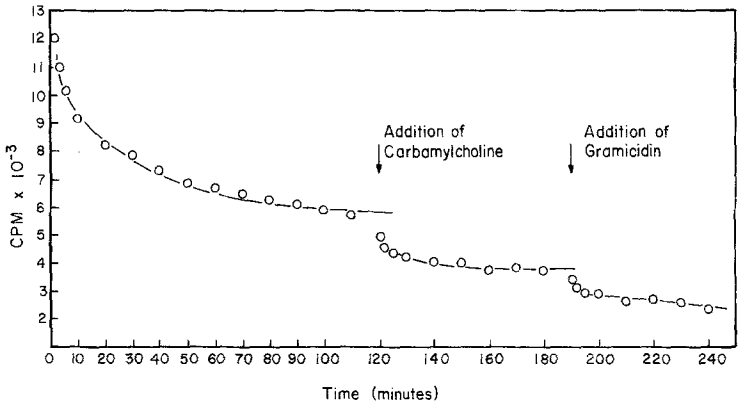

Fig. 5. Complete efflux curve for $^{86}Rb^+$. Vesicles (6.1 mg of protein per ml) equilibrated with sodium eel Ringer's solution were incubated overnight with 100 μCi $^{86}Rb^+$ per ml, before being diluted to a final concentration of 160 mg protein per ml. 0.5-ml aliquots (80 μg protein) were removed and both carbamylcholine (1 mM) and gramicidin (20 μg/ml) were added at the times indicated

Fig. 5, the amplitude difference is still present 1 hr after the addition of carbamylcholine. It is clear that inactivation of the receptor can*not* explain the amplitude difference in the eel vesicles.

One possible explanation of these amplitude differences is the presence of another vesicle population. This population would contain vesicles that are "tight" (i.e., not leaky) but do not contain a functional receptor, or inside-out vesicles in which the receptor binding sites are not accessible to carbamylcholine. Depicted in Fig. 5 is a characteristic efflux curve of vesicles incubated with $^{86}Rb^+$ overnight. The initial efflux (0–120 min) is due to disaggregation of the membrane vesicles as described above and to the exchange of radioactive ions inside the unspecific vesicles with ions in the external medium (Hess et al., 1975). Upon addition of carbamylcholine, an efflux from the specific vesicles is observed (Hess et al., 1975). Addition of gramicidin after ions inside the unspecific and specific vesicles have reached an equilibrium with those in the external medium results in another apparent efflux (Fig. 5). When the vesicles are incubated with radioactive inorganic ions for only 10 min, the final equilibration values reached after dilution of vesicles are the same for carbamylcholine and gramicidin (Fig. 1). This suggests the presence of a nonleaky, unspecific vesicle population (Fig. 5).

To show that gramicidin did not affect the Millipore filter assay, the following experiment was performed: Vesicles were exposed to $^{86}Rb^+$ (50 μCi/ml) for 0.3 min (in the absence of carbamylcholine), before being diluted 10-fold to a protein concentration of 320 μg/ml. The solution was then divided into two: 0.2% ethanol was added to one (control) and gramicidin in ethanol was added to the other to give final concentrations of 20 μg/ml and 0.2% ethanol, respectively. After 30 min, a time period long enough to allow complete exchange of any internal $^{86}Rb^+$ with the external nonradioactive ions, Millipore assays were performed in duplicate using 80 μg of membrane protein per filter. The CPM value obtained reflects the background absorption of $^{86}Rb^+$ by the vesicles and the filter. The results showed that the average CPM per 80 μg protein was approximately the same for the control and gramicidin solutions (429 ± 30 CPM and 418 ± 29 CPM, respectively). Thus, gramicidin *per se* has no effect on the retention of $^{86}Rb^+$ by the membrane vesicles or the filters.

Discussion

It has been shown that in eel electroplax membrane vesicles, the fluxes of inorganic ions are of at least two types (Hess et al., 1975; Hess, Andrews & Struve, 1976): (i) About 85% of the flux is unspecific in that it does not respond to carbamylcholine. The time taken for half of the radioactive ions inside the vesicles to exchange with nonradioactive ions in the external solution, $t_{1/2}$, is on the average 20 min. (ii) About 15% of the flux comes from specific vesicles which do respond to carbamylcholine. In the presence of 1 mM carbamylcholine the exchange of inorganic ions across the vesicle membrane is complete in less than 3 sec (Hess, 1979), and in the absence of carbamylcholine in 20 hr (Hess & Andrews, 1977). In earlier experiments (Kasai & Changeux, 1971*a–c*) the $t_{1/2}$ value for the efflux from the total vesicle population, τ, has been used to assess receptor-controlled fluxes. In the presence of receptor ligand, when the efflux from the specific vesicles is fast, τ will critically depend on the efflux from the unspecific vesicles. The specific vesicles contribute only 15% of the total inorganic ions of the vesicle population; τ is therefore determined by the time it takes for the nonspecific vesicles to lose an amount of radioactive ions which corresponds to 35% (50–15%) of the radioactive ions in the total vesicle population, or about 8 min (Hess et al., 1975, 1976). It can, therefore, be seen why measurements of τ have led to an underestimation of the efficiency of receptor-controlled ion fluxes (Kasai & Changeux, 1971*c*). We have shown previously

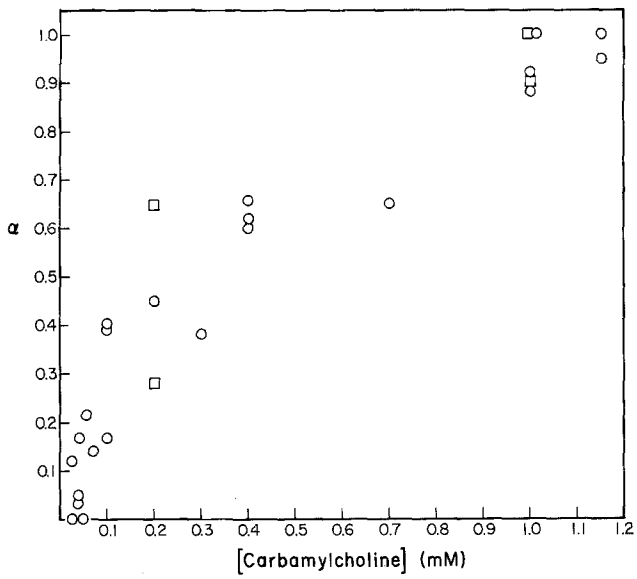

Fig. 6. Corrected plot of the fraction of the ligand-induced $^{86}Rb^+$ efflux due to the fast process (α) *vs.* concentration of carbamylcholine. The raw data were taken from Fig. 4 of Hess et al. (1978). Vesicles incubated overnight with 100 mM KCl, 0.4 M sucrose, 35 μCi of $^{86}RbCl$ per ml, were diluted into a medium containing 100 mM NaCl, 0.4 M sucrose, 1 mM phosphate at pH 7.0 to initiate efflux. After 120 min, carbamylcholine was added

(Hess et al., 1976) why measurements of fluxes from a heterogeneous population of vesicles suggested cooperative effects in receptor-controlled fluxes and why inactivation was not detected in previous flux measurements (Epstein et al., 1980).

Here we identify additional processes which can interfere with measurements of receptor-controlled fluxes in membrane vesicles. In efflux measurements, still routinely used in the qualitative assay of receptor function (Kasai & Changeux, 1971*a–c*; Bernhardt & Neumann, 1978; Hanley, 1978; Hsiao-Ping et al., 1979), disaggregation of vesicles produces artifacts in the Millipore filter assay. In previous experiments we have shown that the receptor-controlled efflux is biphasic; an initial fast phase is followed by a slower phase (Hess et al., 1978). The fraction of the reaction which proceeded by the fast phase α, was found to depend on carbamylcholine concentration (Hess et al., 1978), and had a maximum value of about 0.65. This maximum value of α implied that the ion translocation process associated with the initial fast phase ceased completely and that at some later time a second slow process developed. This led to the interpretation that first the active state of the receptor, and later the inactive (desensitized) state, gave rise to ion channels through the membrane (Hess et al., 1978, 1979). Taking the slowly exchanging ions into account (*see* Section *I*), α reaches a value of 1.0 at high carbamylcholine concentration, and this measurement led to a simpler model, consistent with the results of flow quench experiments, in which only the active state of the receptor forms ion channels (Aoshima et al., 1980; Cash & Hess, 1980). The corrected plot of α *vs.* carbamylcholine concentration is shown in Fig. 6. We conclude, therefore, that inactivation of the receptor does not interfere significantly with the rapid exchange of the vesicle contents at high concentrations of receptor ligand. However, inactivation of the receptor does occur and has been studied (Hess et al., 1978, 1979; Epstein et al., 1980; Cash & Hess, 1980), but the rate of ion movement in the inactivated state of the receptor is greater than was originally thought. The slowly exchanging ions, observed when vesicles are exposed to radioactive ions for longer periods of time, make it difficult to assess the efflux measurements routinely used in qualitative measurements of receptor function (Kasai & Changeux, 1971*a–c*; Bernhardt & Neumann, 1978; Hanley, 1978; Hsiao-Ping et al., 1979).

The apparent discrepancy between the channel formers and the true ionophores in releasing these slowly exchanging ions may be explained by the difference in the mechanism of action of the two types of antibiotics. The true ionophores act by a shuttle mechanism, while the channel formers actually make a pore in the membrane (Pressman, 1976). If we assume that the slowly exchanging ions are trapped in spaces between the lipid bilayers of the vesicle membranes, then we can postulate that they are accessible to true ionophore molecules that can shuttle across the membrane but that they are not accessible to channel formers, such as the acetylcholine receptor, which span the bilayer.

Although gramicidin has been used previously to release radioactive ions from these vesicles, it has not been mentioned that the amplitude of the efflux is greater than with carbamylcholine (Kasai & Changeux, 1971*a*). An amplitude difference in the effects of gramicidin and carbamylcholine has been observed with *Torpedo* receptor-rich membrane vesicles, and it has been suggested that inactivation or desensitization of the receptor is responsible for such amplitude differences (Popot et al., 1976). Here we conclude, however, that the amplitude difference is due to a population of vesicles in which radioactive ions equilibrate slowly and which do not respond to carbamylcholine.

In order to study the underlying mechanism by which ligand binding to the acetylcholine receptor initiates changes in ion permeabilities, it is essential to identify and separate the processes related to the receptor from those which are not. Previous work from this laboratory has separated, both kinetically and physically, the specific, excitable vesicles from

the unspecific, nonexcitable vesicles. This paper has shown that even the specific vesicles have intrinsic properties that are not related to the receptor-mediated kinetics and which must be considered in the measurements.

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