

Reconstitution of a Chemically Excitable Membrane

(acetylcholine receptor/permeability control/membrane self-assembly)

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ABSTRACT Membrane fragments rich in cholinergic (nicotinic) receptor sites, purified from homogenates of *Torpedo marmorata* electric organ, are dissolved in high concentrations of Na cholate and Tris buffer in the presence of Na ethylenediaminetetraacetate, without loss of the ability to bind *Naja nigricollis* [^3H] α -toxin. After extensive dialysis to remove cholate, the solution is supplemented with crude lipids extracted from native membrane fragments and MgCl_2 - CaCl_2 added dropwise to the mixture as a concentrated solution. The reconstituted membranes obtained retain $^{22}\text{Na}^+$. Release of Na from these microsacs increases in the presence of carbamylcholine and the α -toxin from *N. nigricollis* blocks this effect.

The response of an excitable membrane to acetylcholine or its congeners involves two steps, the recognition of the chemical transmitter by the receptor protein and the subsequent movement of cations across the membrane, usually detected in the cell as a decrease of membrane potential or an increase in conductance. By studying release of $^{22}\text{Na}^+$, Kasai and Changeux (1) have shown that membrane fragments purified from homogenates of electric organ of *Electrophorus electricus* still display chemical excitability *in vitro*. These membrane fragments, therefore, contain all the structural elements which are responsible for the response to acetylcholine. We thought that a useful approach to identifying such elements, the ionophore in particular, would be to dissolve the membrane fragments and reassociate them into excitable vesicles.

In an initial part of the work *Electrophorus* membrane fragments were dissolved in deoxycholate and reaggregated into membrane vesicles which retained most of the original α -toxin binding activity of the cholinergic receptor and most of the catalytic activity of acetylcholinesterase (2). In this paper we describe the reconstitution of solubilized membrane fragments from *Torpedo marmorata* electric tissue into sealed microsacs which retain $^{22}\text{Na}^+$ and respond to carbamylcholine by an increased release of $^{22}\text{Na}^+$.

MATERIALS AND METHODS

Preparation of Excitable Microsacs. Membrane fragments from the electric tissue of *Torpedo marmorata* are prepared as described (3), with the alterations included below. Sixty grams of either fresh electric tissue, or of tissue frozen at -20° soon after dissection and thawed before use, are minced with scissors, suspended in 120 ml of 10 mM CaCl_2 , 0.02% NaN_3 , and homogenized at 4° in a Virtis apparatus for 2 min. at maximum speed. The homogenate is sonicated at 4° for three bursts of 10 sec with a 10-sec pause between each burst, with a Branson B-12 Sonifier at force 6. The sonicated homogenate is centrifuged at $5000 \times g$ for 10 min and the supernatant filtered through cheesecloth and then layered on 5 ml of 1.17 M sucrose, 0.02% NaN_3 in Beckman high-speed polycarbonate tubes. The tubes are centrifuged 1.5 hr at 30,000 rpm in

a Beckman type 30 fixed-angle rotor at 4° . The soft pellet is suspended to homogeneity in 3-4 ml of 0.02% NaN_3 , yielding a sucrose concentration of 0.8-1.0 M and 10-25 mg of proteins per ml. The suspension is stored in an ice bath under argon. Fresh tissue yields a small pellet containing 10-100 g of protein and having a specific activity of α -toxin binding sites in the order of 1000 nmol/g of protein. This material thus corresponds to the high specific activity fractions which band between about 1.15 and 1.4 M sucrose (3). Frozen organ yields a larger pellet containing more protein (30-100 mg) and having a specific activity of α -toxin binding sites of 100-300 nmol/g of protein. A similar pellet is obtained with the fresh organ when the homogenate is centrifuged through a layer of 1.17 M sucrose containing 10 mM CaCl_2 . Each of these preparations retains sodium and is chemically excitable (see Results).

Dissolution of Microsacs. Resuspended microsacs are added dropwise with rapid stirring under the surface of a solution containing 11.5 mg of cholate per mg of membrane protein and final concentrations of 0.5 M Tris·HCl, pH 8.0, and 5 mM Na ethylenediaminetetraacetate. For an initial 20 mg of protein the final dissolved volume is about 4.5 ml. The mixture is stirred under argon for 20 min at room temperature, and then centrifuged at $100,000 \times g$ for 1 hr at 4° . Before centrifugation, the only organized structures visible in negatively stained preparations of the solution examined under the electron microscope are collagen-like fibrous structures. The small fibrous pellet obtained after high-speed centrifugation is probably this material. The yields of the dissolution procedure are listed in Table 1.

Reconstitution of Excitable Microsacs. Immediately after centrifugation, the supernatant of the dissolution solution is placed in a 1 mm diameter dialysis tube, tightly tied to prohibit extensive increase in volume and placed in 1 liter of 0.2 M sucrose, 5 mM Tris·HCl at pH 7.5, 0.02% NaN_3 at 4° . The dialysis mixture is stirred under argon, at 4° with changes of the outside solution every 12 hr. At 48 hr the now slightly turbid solution is removed from the dialysis bag and placed in a small beaker under argon and a suspension of sonicated lipids extracted from *Torpedo* microsacs (see below) is added. After mixing at 4° for 0.5 hr, addition of a solution of 1 M MgCl_2 is begun. The salt is added in 10 portions at 15-min intervals in geometrically increasing amounts (from 1/1024 to $1/2$ of the total to give final concentrations of 25 mM MgCl_2 and 2.5 mM CaCl_2 . After a total of 3 hr of stirring the now turbid suspension is layered on 1.5 ml of 1.17 M sucrose in the bottom of one or two Beckman high-speed polycarbonate tubes. After centrifugation for 1.5 hr at 40,000 rpm in a Beckman type 40 rotor ($106,000 \times g$) at 4° , material is found at the interface between the sucrose and the sample volume, suspended in the sucrose layer, and in a soft pellet. The top

TABLE 1. Summary of dissolution and reconstitution of *Torpedo microsacs*

	Volume (ml)	Protein		[³ H]α-toxin sites		Acetylcholine esterase		Phospholipid	
		(mg/ml)	(% yield)	(nmol/g of protein)	(% yield)	(nmol/g of protein)	(% yield)	g/g of protein)	(% yield)
High speed pellet (native vesicles)	3.0	30.3	100	220	100	3.45	100	0.36	100
Dissolved vesicles	6.9	13.6	103	380	178	1.50	45	0.41	117
Dissolved vesicle supernatant	6.55	14.0	101	325	155	1.31	39	0.36	99
Dialyzed suspension	8.45	7.7	71.5	183	61	1.85	39	0.25	47
Reconstituted vesicles	15.5	4.1	70	249	79	1.80	37	0.30	57*
Light fraction	1.7	3.4	6.5	315	9.3	5.30	10	0.92	16
Pellet fraction	12.0	2.7	36	430	70	1.80	19	0.25	25

* This value includes 4.3 mg (13% of the original total) of sonicated phospholipid added after dialysis. The high-speed pellet contained 20% of the protein, 65% of the receptor, 19% of the esterase and 49% of the phospholipid found in the low-speed supernatant from which it came. Details of the assays used and of the reconstitution procedure are described in *Materials and Methods*.

band is often aggregated into large lumps and has a high content of lipid phosphate (Table 1). The pellet is the reconstituted material. The reconstituted material is resuspended to homogeneity, with a tissue homogenizer if necessary, and then stored on ice under argon. If the reconstituted material is centrifuged into a sucrose gradient like that used to prepare native microsacs (3) the gradient profile is similar to that for native material with the addition of a layer of "high-lipid" material on top of the gradient. Table 1 gives a summary of the various stages of the reconstitution procedure.

Measurement of ²²Na⁺ Efflux. Sodium efflux is assayed by the procedure for *Electrophorus* microsacs (1). The microsac suspension is incubated on ice from 8 to 20 hr (to allow complete equilibration between the inside and the outside of the microsacs) with 10 mM NaCl containing enough ²²NaCl (prepared by the Radiochemical Centre, Amersham, Great Britain) to give about 3 × 10⁷ cpm/ml in 0.5 M sucrose, 0.02% NaN₃. At zero time an aliquot of the mixture (50–250 μl) is diluted 100-fold into an ice-cold salt solution with an ionic strength close to that of *Torpedo* Ringer's solution but without the urea (255 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 1.5 mM phosphate buffer, pH 7.0) and containing or not a cholinergic effector, usually carbamylcholine, an agonist not degraded by acetylcholinesterase. The osmolarity of the incubation and dilution solutions are almost identical. The diluted suspension is gently stirred and then incubated in an ice bath. Samples (0.5- to 5-ml) are filtered at various times through 0.45-μm HWAP Millipore filters presoaked in the dilution medium, which are then dried and the radioactivity retained determined by scintillation counting. The filtration and wash usually require 30 sec, but some preparations filter slowly (up to 90 sec). A sample is taken 8–20 hr after dilution in order to determine the amount of radioactivity trapped on the filter at equilibrium.

In the case of *Electrophorus* microsacs, the equilibrium quantity is that expected from the apparent volume of the suspension and the dilution factor. With both native and reconstituted *Torpedo* microsacs this is not the case. About 25% of the counts retained at zero time are still trapped on the filter after equilibration following the 100-fold dilution of native microsacs. This value is as high as 50% for reconstituted sacs.

The level of this background does not vary by more than 20% for a given preparation and decreases slowly with extended incubation (to approximately 10% of its initial value after 60 hr at room temperature). Influx experiments indicate that these counts are trapped by the microsacs filtered immediately upon addition of radioactive sodium.

The radioactivity of a 10-μl aliquot of the diluted suspension is determined for each condition tested in a given experiment and the raw data are always normalized to the same level of radioactivity, that is to the same amount of incubation suspension initially added. In general the correction factor does not exceed 5%. The radioactivity trapped at equilibrium is then subtracted as background from each time point to obtain the level of exchangeable sodium retained by the microsacs. With the use of vesicles which had been dissolved and reconstituted in the presence of [¹⁴C]cholate of high specific activity and had cholate incorporated into them, it was possible to demonstrate that less than 5% of this radioactivity passed through the Millipore filter and that there was less than 5% variation in radioactivity retained for aliquots filtered at different times or in the presence or absence of carbamylcholine.

The effect of α-toxin on ²²Na⁺ efflux is determined after a 1 hr preincubation on ice of the suspension with 1 μM α-toxin. The microsacs have an apparent volume (V_{app}), expressed as μl of exchangeable solution per mg of membrane protein as determined by the amount of exchangeable radioactivity retained by the suspension at zero time of dilution. V_{app} ranges, in general, between 0.1 and 0.4 μl/mg of proteins. Thus about 50 μg of membrane protein filtered for each time point gives a reasonable level of retained radioactivity. Excitability is expressed as $(\tau_0/\tau) - 1$ where τ_0 and τ are the times for half equilibration of ²²Na⁺ between the inside and the outside of the microsacs in the absence and in the presence of a cholinergic effector, respectively.

Extraction of Lipid. Pelleted native microsacs prepared from 60 g of organ are resuspended in 5 ml of H₂O, then 10 ml of CH₃OH and 5 ml of CHCl₃ are successively added. The mixture is stirred for 15 min at room temperature and centrifuged 15 min at 4000 × g. Twenty milliliters of CHCl₃ and 20 ml of H₂O are added to the supernatant, and 5 ml of CH₃OH and 10 ml of CHCl₃ added to the pellet resuspended in 5 ml of H₂O.

After stirring for 15 min at room temperature and centrifugation at $4000 \times g$ for 45 min, the bottom (chloroform) layers are pooled, dried in a rotatory evaporator and resuspended in about 1 ml of CHCl_3 . This preparation, containing between 15 and 20 mg of phospholipid is stored at -20° under argon.

A few hours before lipid is to be used in a reconstitution experiment, a volume of the CHCl_3 solution containing a quantity of phospholipid equivalent to 20% of the weight of membrane protein used for the reconstitution is evaporated under argon. After evaporation enough reconstitution dialysis buffer is added to give a phospholipid concentration of less than 1.5 mg/ml. The mixture is sonicated in an ice bath under argon, for 3×3 -min periods with a Branson B-12 sonifier equipped with a microtip. Between each period there is a 1-min pause for cooling. The sonicated suspension, now a cloudy blue, is centrifuged 1 hr at $100,000 \times g$. Some material pellets, and a small amount floats to the top. The "blue" solution of the supernatant, containing about 80% of the phospholipid originally added, is used for the reconstitution.

[^3H] α -Toxin Binding. Cholinergic receptor sites were assayed with α_1 -isotoxin purified from the venom of *Naja nigricollis* and tritiated by the method of Menez *et al.* (4). Binding of [^3H] α -toxin to microsacs is assayed by Millipore filtrations (5). Under the conditions used [^3H] α -toxin binds exclusively to the cholinergic receptor site (5). In general 0.6 pmol of 10.5 Ci/mmol of [^3H] α -toxin are titrated by prolonged incubation (4 hr) at room temperature with increasing amounts of membrane suspension in the presence of 250 mM NaCl, 4 mM CaCl_2 , 2 mM MgCl_2 , 5 mM Na phosphate buffer, pH 7.0, followed by filtration through a Millipore 0.45- μm filter and washing with 20 ml of 4° Ringer's solution. When solubilized receptor is assayed, "helper" protein (6) is added to the incubation mixture to allow retention of the receptor by the filters. Radioactivity retained by the filters is determined, after drying, by scintillation counting. With freshly labeled toxin solutions in the absence of membrane fragments, less than 10% of total counts stick to the filters, while in the presence of excess membrane fragments, 100% of the counts are retained.

Protein concentration is determined with bovine-serum albumin as the standard (7). Acetylcholinesterase (EC 3.1.1.7) is assayed with acetylcholine as substrate (8). To convert the change in optical density per time per volume of membrane suspension into standard units, it is assumed that acetylcholinesterase has a molecular weight of 260,000 and the pure enzyme a turnover number for acetylcholine of 750 mol/hr per g of proteins (9). Lipid-phosphate is determined (10) and the mass of total phospholipid calculated by assuming an average phospholipid molecular weight of 800. The lipid-phosphate in dissolved and reconstituted samples is determined by extracting approximately 500 μg of membrane protein in 0.5 ml of H_2O with 0.5 ml of CH_3OH and 1 ml of CHCl_3 , and assaying phosphate in the CHCl_3 phase.

All solutions were made with double-distilled water.

RESULTS

Membrane fragments rich in α -toxin binding sites and thus in cholinergic receptor sites (5) are purified by ultracentrifugation from a crude homogenate of *Torpedo* electric organ. These membrane fragments retain $^{22}\text{Na}^+$ and thus consist of closed vesicles or microsacs (Fig. 1). The apparent volumes (see *Methods*) of the microsacs usually range between 0.1 and 0.5 $\mu\text{l}/\text{mg}$ of proteins, while the times for half equilibration of $^{22}\text{Na}^+$ (τ_0) (see *Methods*) range between 5 and 25 min. In the

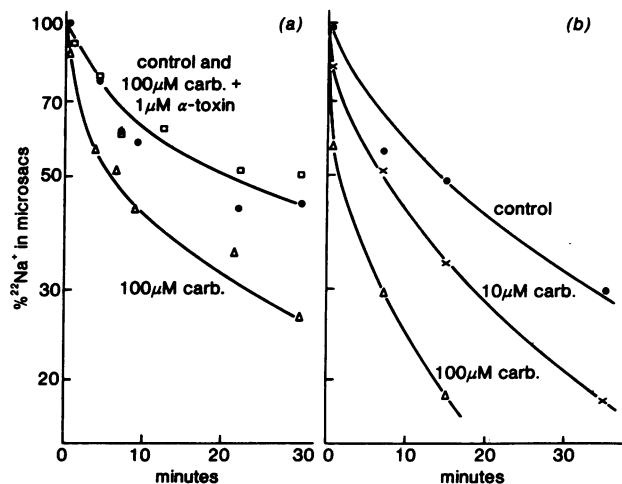


FIG. 1. $^{22}\text{Na}^+$ efflux from native *Torpedo* microsacs about 24 hr after fractionation. Physiological *Torpedo* saline (\bullet), *id.* + 100 μM carbamylcholine (carb.) (Δ), *id.* + 10 μM carbamylcholine (\times), or *id.* + 100 μM carbamylcholine after preincubation in 1 μM α -toxin (\square). The microsacs in (a) contained 150 nmol of α -toxin-binding sites per g of protein and had a V_{app} of 0.16 $\mu\text{l}/\text{mg}$ of protein. The 100% value was 1000 cpm. The respective values for (b) were 1400 nm, 0.3 $\mu\text{l}/\text{mg}$, and 800 cpm. Details of the assay are described in *Materials and Methods*.

presence of 100 μM carbamylcholine, a compound known to enhance the permeability of the electroplax membrane (11), i.e., an agonist, the efflux of $^{22}\text{Na}^+$ is faster (Fig. 1). This effect is blocked by the α -toxin from *N. nigricollis* (Fig. 1a). The microsacs derived from the electric organ of *Torpedo* thus display chemical excitability *in vitro*.

The time course of $^{22}\text{Na}^+$ release from *Torpedo* microsacs and the amplitude of the response to carbamylcholine [$(\tau_0/\tau) - 1$] vary from preparation to preparation. Maximum responses are observed when $^{22}\text{Na}^+$ efflux at rest, that is, in the absence of a cholinergic effector, is slow. When the resting efflux is rapid, which is sometimes the case for a fresh preparation and always the case for an old preparation, the response is small and sometimes not detectable.

Storage of the microsac preparation in 0.5 M sucrose, 0.02% NaN_3 at 0° results in a decrease of excitability caused by an increase in the rate of Na^+ release at rest with little change in the rate of Na^+ release in the presence of 100 μM carbamylcholine (Fig. 2). After several days, the two rates coincide and excitability is lost, even though a significant amount of toxin-binding activity remains in the preparation.

Dissolution of *Torpedo* microsacs occurs in high concentrations of Na cholate and Tris buffer in the presence of Na ethylenediaminetetraacetate (see *Materials and Methods*). From this solution, less than 10% of the membrane protein is pelleted by centrifugation at $100,000 \times g$ for 1.5 hr (Table 1). Some toxin-binding sites, therefore, either are shielded on the inside of inverted native microsacs or are inaccessible to the α -toxin in some other way. In contrast, during the same treatment, the activity of acetylcholinesterase decreases, perhaps because of an effect of cholate on the stability of the protein.

As the first step in the reconstitution of excitable membrane vesicles from dissolved microsacs, cholate is removed by dialysis of the solution (approximately 5 mg of protein per ml) against a Tris-sucrose- NaN_3 buffer for 48 hr. [^{14}C]Cholate leaves the bag at a constant rate with a half-time of 7-8 hr. After 48 hr less than 1% of the [^{14}C]cholate originally present

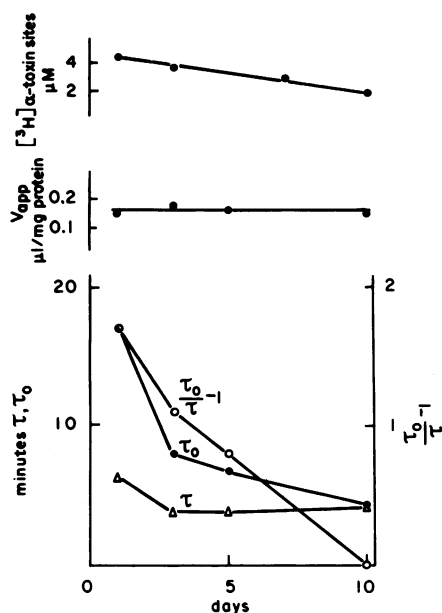


FIG. 2. Decay of excitability in native *Torpedo* microsacs. The lower plot shows τ_0 for microsacs diluted into *Torpedo* saline solution (●) or *id.* + 100 μM carbamylcholine (Δ) and the excitability value $(\tau_0/\tau) - 1$ (○).

remains in the dialysis bag. The rate of loss then changes to a half time of about 17 hr. Even though significant amounts of cholate are still present after 48 hr of dialysis, further dialysis does not improve the results of the reconstitution. Dialysis for 140 hr reduces the cholate concentration to less than 0.1% of the initial value but, by that time, much of the toxin-binding activity has been lost.

A slight loss of protein and α -toxin-binding activity occurs during dialysis, but there is a greater loss of CHCl_3 - CH_3OH extractable phosphate, i.e., phospholipid (Table 1). This is perhaps why the dialyzed material cannot be induced to form consistently sealed vesicles. To obtain microsacs which finally retain $^{22}\text{Na}^+$, we add, after dialysis, extra lipid prepared from a CHCl_3 - CH_3OH extract of *Torpedo* membrane fragments. The optimal amount of phospholipid added corresponds to 20% of the weight of membrane proteins present in the dissolved microsacs (see *Materials and Methods*).

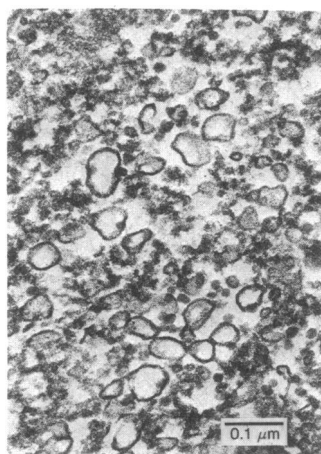


FIG. 3. Thin section of a pellet of reconstituted microsacs. The pellet was fixed in 1% glutaraldehyde- OsO_4 , embedded in "vestopal W" and stained with Pb citrate and UO_2 acetate.

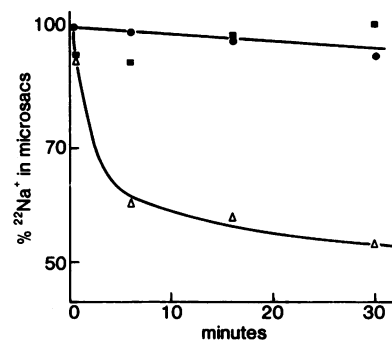


FIG. 4. $^{22}\text{Na}^+$ efflux from reconstituted *Torpedo* microsacs 10 hours after reconstitution. *Torpedo* saline solution (●), *id.* + 100 μM carbamylcholine (Δ), or *id.* + 100 μM carbamylcholine after preincubation in 1 μM α -toxin (■). The reconstituted vesicles contained 270 nmol of α -toxin-binding sites per g of protein and had a V_{app} of 1.3 $\mu\text{l}/\text{mg}$ of protein. The 100% value was 450 cpm.

The dialyzed material is visually turbid and thus has aggregated as the concentration of cholate was reduced, but this material does not retain sodium. After addition of MgCl_2 and CaCl_2 , $^{22}\text{Na}^+$ is retained by the material, and thus sealed vesicles are formed. The way salt is added seems critical for a successful reconstitution. If the salt concentration is increased by dialysis, the material which aggregates does not always retain $^{22}\text{Na}^+$. However, if a concentrated salt solution is added dropwise to the dissolved membranes supplemented after dialysis with phospholipids, a consistent retention of $^{22}\text{Na}^+$ by the reconstituted material is observed (see *Methods*).

When the total amount of material is small, i.e., 15 mg of protein instead of the 50–100 mg often used, lumps appear during salt addition. As long as the reconstituted material can be resuspended to homogeneity by a tissue homogenizer, $^{22}\text{Na}^+$ retention and excitability appear unaffected.

Centrifugation of the reaggregated material in a sucrose gradient gives two major fractions which differ in their lipid to protein ratios, in their content of acetylcholinesterase and receptor, and in their excitability. A light fraction bands at the interface between the sample (in 0.2 M sucrose) and the underlying more concentrated solution of sucrose. The rate of $^{22}\text{Na}^+$ efflux from this material is as slow as that from the most impermeable reconstituted microsacs, but does not change in the presence of either carbamylcholine or α -toxin. The lipid to protein ratio is higher than for native microsacs (Table 1) and the amount of [^{14}C]cholate per g of protein present after dialysis is about twice as large as the other fraction.

The second fraction enters a sucrose gradient and bands at a concentration between 1.0 and 1.4 M. When a layer of 1.17 M sucrose is used instead of a gradient, the material makes a soft pellet at the bottom of the centrifuge tube. These reconstituted vesicles have a phospholipid to protein ratio smaller than that of the native microsacs. In agreement with the previous observation (2) the specific activity of toxin-binding sites is higher in the reconstituted material than for native microsacs, but the reverse is true for acetylcholinesterase. The reconstituted microsacs retain $^{22}\text{Na}^+$ (Fig. 3) and have apparent volumes ranging mostly from 0.1 to 0.4 $\mu\text{l}/\text{mg}$ of proteins. The electron micrograph of a thin section of reconstituted microsacs shown in Fig. 3 reveals vesicles limited by a single membrane of about 70 Å average thickness. Their diameter ranges from 30 to 100 nm. Interspersed

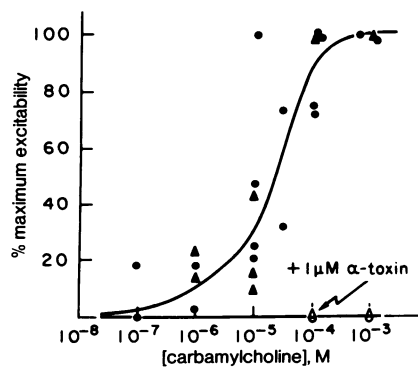


FIG. 5. Effect of carbamylcholine on the efflux of sodium from native (●) and reconstituted (▲) *Torpedo* microsacs. The excitability $(\tau_0/\tau) - 1$ is expressed as a percentage of the maximum response for a given preparation, a value which varied from 1 to 8. Four native and four reconstituted preparations are represented.

between the vesicles is amorphous material which adsorbs stain but does not exhibit a typical membrane structure (2).

The $^{22}\text{Na}^+$ release from reconstituted microsacs is faster in the presence of carbamylcholine and this effect is blocked by $1 \mu\text{M}$ α -toxin (Fig. 4). Thus the reconstituted microsacs are excitable.

As for native microsacs, the amplitude of the response to carbamylcholine depends on the value of the rate of $^{22}\text{Na}^+$ release at rest. Some reconstituted microsacs release $^{22}\text{Na}^+$ more slowly than native vesicles and thus appear very excitable. Again in agreement with observations made with native microsacs, the rate of $^{22}\text{Na}^+$ release increases with time of storage to finally coincide with the efflux measured in the presence of carbamylcholine. This decay of excitability is usually much faster for reconstituted than for native vesicles.

The time course of $^{22}\text{Na}^+$ release from reconstituted vesicles in the presence of carbamylcholine often seems to consist of two phases: first a rapid loss and then a slow decrease which parallels the resting efflux (Fig. 3). This phenomenon presumably reflects a heterogeneity of the population of microsacs, only a fraction of them being sensitive to carbamylcholine.

Kasai and Changeux (1) were able to construct dose-response curves to cholinergic agonists following $^{22}\text{Na}^+$ efflux from *Electrophorus* microsacs. These curves closely parallel those obtained by following the steady-state potential recorded from isolated electroplax upon bath application of cholinergic agonists. We have constructed a similar curve for the effect of carbamylcholine on $^{22}\text{Na}^+$ release at 0° from native and reconstituted *Torpedo* microsacs (Fig. 5). For both types of material the carbamylcholine concentration which causes the half-maximal effect is, at 0° , between 10 and $100 \mu\text{M}$. It is more difficult to obtain precise efflux measurements with *Torpedo* than with *Electrophorus* vesicles and thus the dose-response curve for carbamylcholine contains a significant amount of scatter. Nevertheless, carbamylcholine seems to affect Na^+ release from both native and reconstituted microsacs to a similar extent, which suggests that the control of Na^+ efflux by the cholinergic receptor functions in both systems. The ionic selectivity of the observed effect in reconstituted microsacs remains to be studied.

DISCUSSION

Recovery of membrane-dependent functions upon reconstitution of a membrane structure has been recently reported for a number of systems (12-15).

We have defined experimental conditions in which carbamylcholine causes an increase of passive Na^+ release from reconstituted microsacs. Receptor and ionophore must, therefore, spontaneously orient in the reformed membrane in such a way that (1) a significant fraction of the cholinergic receptor sites is accessible to carbamylcholine present in the surrounding solution, (2) the ion path spans the membrane to open on both sides, and (3) the regulatory coupling between receptor and ionophore is functional. This does not imply that the native structure of the biological membrane is recovered by reconstitution. The different ratios of lipid to protein and of acetylcholinesterase to cholinergic receptor for native and reconstituted microsacs suggest the opposite.

But the fact that a response to cholinergic agonists is recovered, even though the reconstituted membranes differ in some ways from the native ones, suggests that only a limited number of structural characteristics of the native membrane are critical for excitability. Fractionation of the dissolved membrane before reconstitution should establish what components are critically required and particularly whether the purified receptor protein alone provides the ion path controlled by the cholinergic receptor site.

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