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ABSORPTION FILTRATION

A TOOL FOR THE MEASUREMENT OF ION TRACER FLUX IN NATIVE MEMBRANES AND RECONSTITUTED LIPID VESICLES

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A rapid, simple and reproducible method has been developed for the measurement of ion tracer flux with both native membrane vesicles and reconstituted lipid vesicle systems. Following the absorption of vesicles onto glass fiber filters, tracer flux is performed directly upon the deposited samples. In contrast to the more conventional vacuum and gel filtration techniques, absorption filtration exhibits comprehensive data retrieval whereby the removal of extravesicular ions, the retention of intravesicular ions and the amount of ions fluxed can be accurately analyzed. Both influx and efflux assays have been designed to measure the carbamylcholine-induced flux of $^{22}Na^+$ which is characteristic of acetylcholine receptor-enriched membranes from *Torpedo californica* electroplax. The flux signal-to-background noise ratio is maximized in the efflux assay, since agonist activation is performed subsequent to the exhaustive removal of extravesicular tracer. An interesting feature of the influx assay is that the agonist-induced uptake of $^{22}Na^+$ can be repeated with the original vesicles which additionally maximizes the flux signal. With either approach, the inactivation of ionophoric activity due to prolonged exposure to agonists ('desensitization') can be reversed upon removal of agonist without dilution of the deposited samples. Due to the large array of glass fiber filters and ion-exchange disks, the absorption filtration technique should be able to accommodate the transport and binding of soluble molecules to a variety of intact cells, membranes and reconstituted lipid vesicles.

Introduction

The acetylcholine receptor performs an essential role during postsynaptic depolarization at the vertebrate neuromuscular junction and in the electroplax organ of a variety of fish (reviewed in Ref. 1). This drug-receptor phenomenon is mediated by (i) the specific interaction of the neurotransmitter (acetylcholine) with the membrane-bound receptor, followed by (ii) an increase in the ion permeability of the postsynaptic membrane. Experimental quantitation of acetylcholine receptor functionality thus requires the precise monitoring of ion translocation in either native or reconstituted membrane preparations. To investigate these events, a number of approaches have been developed which measure the influx and efflux of radiolabeled tracer cations [2–5]. Similar approaches have been applied to assess ionophore activity in other membrane systems [6,7].

We presently describe an absorption filtration technique for the measurement of ion tracer flux in both native receptor-enriched membranes and reconstituted receptor vesicles. It is partially based upon (i) the 'hygroscopic desorption' technique of Conrad and Singer [8] for measuring the binding

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of amphiphatic compounds to cell membranes and phospholipid vesicles, and (ii) the 'serial washing' procedure of Thomas et al. [9] for the isolation of DNA-protein complexes. Absorption filtration is also comparable to the controlled elution technique developed by Grunhagen [7] except that the latter requires a programmable pipet, pulse generator and an electronic circuit to integrate the components. Conversely, absorption filtration requires minimal experimental setup (filters and pipet) without sacrificing accuracy or reproducibility. Unlike vacuum filtration [2], gel filtration [6] and ion-exchange chromatography techniques [3], absorption filtration permits an accurate determination of both extravesicular and intravesicular ions.

In this communication, we examine the ion translocation properties of native acetylcholine receptor-enriched membranes prepared from *Torpedo californica* electroplax. For comparative analysis we additionally examine ion flux in lipid vesicles assembled either with or without purified receptor [10]. We do not intend to present a comprehensive treatise on acetylcholine receptor functionality, but rather utilize the well-accepted properties of membrane-bound receptor in order to demonstrate the utility of the absorption filtration technique. To this end we have developed an efflux and influx assay to measure the agonist-induced translocation of $^{22}Na^+$ characteristic of functional receptor-enriched membranes. Absorption filtration should prove quite useful for the measurement of ion tracer flux in a variety of cell membranes and reconstituted vesicle systems.

Materials and Methods

Materials. Torpedo californica were obtained live from Pacific Biomarine Supply Co. and octylglucopyranoside was purchased from Calbiochem. α-Bungarotoxin was purified from *Bungarus multicinctus* venom (Sigma) following published procedures [11]. ^{125}I-Labeled α-bungarotoxin, ^{22}NaCl, and cholesteryl[1-^{14}C]oleate were purchased from New England Nuclear. α-Cobratoxin was purified from *Naja naja siamensis* venom (Sigma) by the method of Ong and Brady [12]. GF/A, GF/C, GF/D, 934-AH and DE81 filter disks were obtained from Whatman.

Preparation of membrane-bound and purified acetylcholine receptor. Receptor-enriched membranes were prepared from freshly dissected electroplax tissue by the method of Delegeane and McNamee [13]. The membranes routinely exhibited a specific activity of approx. 1.5 nmol of α-bungarotoxin bound per mg protein and were stable with regard to α-toxin binding and flux activity for 2 to 3 months when stored frozen in liquid nitrogen.

The acetylcholine receptor was solubilized from crude electroplax membranes with 34 mM octylglucopyranoside [14] and was purified by α-cobratoxin affinity chromatography as previously described [15], except that octylglucopyranoside replaced Triton X-100 throughout the procedure. The specific activity of the purified receptor was equal to or greater than 9 nmol of α-bungarotoxin bound per mg protein. Radiolabeled α-bungarotoxin binding to the receptor protein was determined using a DEAE-cellulose (DE81) filter disk assay procedure [16] and protein concentration was determined by the method of Lowry et al. [17]. Total lipids from crude electroplax membranes were extracted as recently described [18] and lipid phosphorus was determined by a modification of a published procedure [19].

Reconstitution procedure. Octylglucopyranoside and total electroplax lipid in a 4/1 (w/w) ratio were dissolved in benzene and appropriate aliquots of the mixture were transferred to test tubes and lyophilized overnight. Purified receptor (0.14–0.36 mg/ml) was added to the detergent/lipid mixtures with lipid to protein ratios ranging from 48/1 to 69/1. The detergent/lipid/receptor protein mixed micelles were dialyzed against reconstitution buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 0.02% NaN_3) until greater than 99% of the octylglucopyranoside was removed as estimated by using the radioactive tracer, octyl [U-^{14}C]glucopyranoside. Plain lipid vesicles were prepared in a similar manner except that reconstitution buffer instead of receptor protein was added to the detergent/lipid mixtures. In order to estimate the recovery of total lipid upon either dialysis or absorption filtration, an aliquot of cholesteryl [1-^{14}C]oleate as a lipid tracer was also added to the detergent/lipid mixtures.

Absorption filtration. (1) Serial washing. Whatman 934-AH glass fiber disks (2.4 cm diameter)

with the rough surfaces facing upward were placed in a row on parafilm which was positioned on a flat surface. A double layer of Whatman GF/C glass fiber filters cut into 7 mm diameter disks, also with the rough surfaces facing upward, were centered onto the first 934-AH disk. The upper GF/C disks were first presoaked with 10 μl of the appropriate buffer, followed by the addition of 10 μl of the required sample to the center of the disks. The deposited samples were carefully washed dropwise with 15 μl buffer in a manner such that each drop of buffer covered the surface of the upper GF/C disks before being absorbed into the underlying 934-AH disk. With clean forceps, the GF/C disks were transferred onto the next 934-AH disk and washed again with 15 μl buffer. For serial washing experiments, the wash and transfer cycle was repeated 10 to 20 times. Other filter types utilized as the upper disks in certain experiments were GF/A, GF/D, 934-AH glass fiber filters and DE81 ion-exchange filters.

The serial washing procedure was particularly useful in analyzing the separation of extravesicular $^{22}Na^+$ from the intravesicular pool. After the final wash and transfer cycle, the total $^{22}Na^+$ radioactivity is calculated by summing the radioactivities associated with the upper GF/C disks and each underlying 934-AH disk. The radioactivity associated with the first 934-AH disk is subtracted from the total and represents the amount of $^{22}Na^+$ retained on the upper GF/C disks after the initial 15 μl wash. The amount of $^{22}Na^+$ retained after each wash and transfer cycle is then calculated with the last value equaling the amount of radioactivity actually measured on the GF/C disks. The fraction of radioactivity retained at each wash cycle is then calculated and plotted versus the volume of washing buffer in 15 μl increments (Figs. 1, 2, 4). The retention of electroplax lipid vesicles following absorption filtration was analyzed in a similar manner except that radioactivity due to the cholesteryl[1-^{14}C]oleate lipid tracer was measured on all the filter disks. For the retention of receptor-enriched membranes, lipid phosphorus was directly determined only on the upper disks after three 100 μl wash and transfer cycles.

(2) Efflux. Native receptor-enriched membranes (approx. 5 mg/ml protein) were equilibrated overnight with 75 μCi/ml $^{22}Na^+$. After depositing the $^{22}Na^+$-equilibrated samples on the upper GF/C disks, the samples were washed with three 100 μl wash and transfer cycles instead of the ten 15 μl cycles described above for the serial washing procedure. This modification economized the number of 934-AH filters utilized and reduced the assay time for routine efflux experiments while giving essentially the same results obtained by serial washing. After the washing cycles, all of the apparent extravesicular $^{22}Na^+$ was washed into the three 934-AH disks, whereas the intravesicular $^{22}Na^+$ remained with the deposited samples on the GF/C disks. In the efflux cycle, the GF/C disks were transformed to a remaining 934-AH disk and washed with 50 μl buffer with or without carbamylcholine.

After equilibration with $^{22}Na^+$, the receptor-enriched membranes could be completely desensitized by preincubation with carbamylcholine ($1 \cdot 10^{-5}$ M) for 30 min. Agonist-induced efflux was assayed as described above except that the buffer used during the washing cycles also contained the same concentration of carb used to desensitize the membranes. When the washing buffer did not contain the agonist, desensitization was completely reversed when $1 \cdot 10^{-4}$ M carbamylcholine or less was used in the initial preincubation.

(3) Influx. The receptor-enriched membranes were deposited on the upper GF/C disks prior to a 10 μl addition of 75 μCi/ml $^{22}Na^+$ with or without carbamylcholine. The membranes were then washed with three 100 μl wash and transfer cycles as described above for the efflux assay. The radioactivity remaining with the GF/C disks at the termination of the washing cycles was taken to be the amount of intravesicular $^{22}Na^+$. Background levels of $^{22}Na^+$ were determined either by conducting the influx assay in the absence of membranes or by ading the $^{22}Na^+$ prior to the addition of membranes.

Following the termination of certain influx experiments, an additional dose of $^{22}Na^+$ with or without the same concentration of carbamylcholine was added to the same membranes followed by the three 100 μl wash cycles. When membranes were to be desensitized, 10 μl of carbamylcholine was added to the membranes immediately preceding the addition of $^{22}Na^+$ which contained saturating levels of the agonist ($0.5 \cdot 10^{-3}$ M) normally

sufficient to elicit maximum agonist-induced tracer influx.

Results

Salient features of absorption filtration

The retention of electroplax lipid vesicles and native receptor-enriched membranes on DEAE-cellulose (DE81) disks and various glass fiber disks (GF/A, GF/C, GF/D and 934-AH) following absorption filtration was examined (Table I). Lipid vesicles, assembled by detergent-dialysis either with or without receptor protein, are best retained on upper GF/C type disks with greater than 0.97 fraction remaining after three 100 μl wash and transfer cycles. Other filter types utilized as the upper disks are approx. 20 to 30% less efficient than GF/C disks in retaining the lipid vesicle preparations. Three glass fiber filter types (GF/A, GF/C and 934-AH) are equally effective in retaining native receptor-enriched membranes with about 0.85 fraction of sample remaining. Of the various glass fiber filter types, we have concentrated our attention on the GF/C disks and have compared the results obtained in various experiments with those obtained with the DE81 ion-exchange disks.

Once absorbed onto either GF/C or DE81 filters, lipid vesicles appear to adhere very tightly. Increasing the salt concentration of the washing buffer to 500 mM NaCl causes at best a 10% decrease in the retention of lipid vesicle preparations after three 100 μl wash cycles (data not presented). Similarly, washing with 15 mM octylglucopyranoside exerts no effect on the retention of the lipid vesicles, whereas it is sufficient to induce a dramatic increase in the release of intravesicular ions [10]. A significant decrease in the retention of the lipid vesicles does occur when the octylglucopyranoside concentration is increased to 34 mM which causes maximal solubilization of electroplax membranes [14].

We have also observed that lipid vesicles assembled with either soybean phospholipid or total electroplax lipid adhere similarly to DE81 disks (data not shown). Conversely, soybean phospholipid vesicles are retained to a much lesser degree than electroplax lipid vesicles on GF/C disks. Only soybean phospholipid vesicles which contain receptor protein are significantly retained on GF/C disks with the amount of retention proportional to the amount of receptor reconstituted. The exclusion of protein-free soybean phospholipid vesicles from GF/C disks is convenient when flux data pertaining solely to protein containing vesicles are desired. When measurements reflecting the total vesicle population are required, DE81 disks rather than GF/C disks should be utilized.

The serial washing profiles of reconstituted receptor vesicles and receptor-enriched membranes after equilibration with $^{22}Na^+$ are shown in Fig. 1. The retention of the lipid vesicles as estimated with a radioactive lipid tracer is also shown for comparative analysis. In the absence of membrane or lipid vesicle samples, free $^{22}Na^+$ is exhaustively removed from upper GF/C disks with about 0.05 fraction remaining after two 15 μl wash cycles and less than 0.001 fraction remaining at the completion of the assay (Fig. 1A). After equilibration of $^{22}Na^+$ with either membrane or lipid vesicles, there is a significant plateau in tracer removal which reflects the retention of intravesicular $^{22}Na^+$ on the upper GF/C disks. Reconstituted receptor vesicles, which are normally assembled with approximately 50-fold higher lipid/protein (w/w) ratios than receptor-enriched membranes, also re-

TABLE I

FRACTION SAMPLE RETAINED

Fraction of sample retained on upper filter disks after three 100 μl wash and transfer cycles. Values are expressed as either single determinations or averages of two or more determinations with respective standard deviations.

Upper filter type	Fraction sample retained		
	Reconstituted receptor vesicles [a]	Plain lipid vesicles [a]	Receptor-enriched membranes [b]
DE81	0.68 ± 0.04	0.74 ± 0.05	0.57
GF/A	0.69	0.79	0.85 ± 0.01
GF/C	0.99 ± 0.01	0.97 ± 0.01	0.85 ± 0.01
GF/D	–	–	0.57 ± 0.05
934-AH	0.74	0.79	0.86 ± 0.05

[a] Estimated by measuring the amount of cholesteryl[1-^{14}C]oleate retained.

[b] Estimated by measuring directly the amount of membrane lipid phosphorus retained on the upper disks.

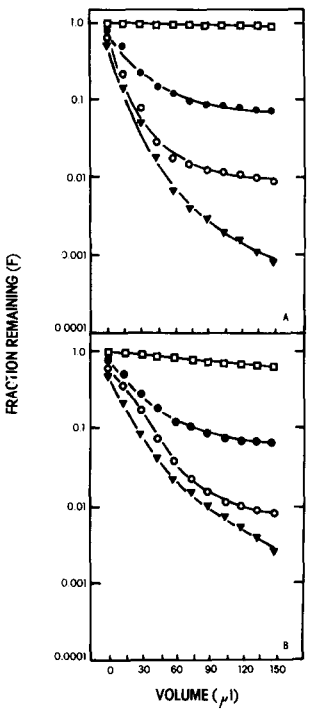

Fig. 1. Serial washing diagram depicting the fraction of ion or lipid tracer remaining (presented on a logarithmic scale) versus each 15 μl wash and transfer cycle. Samples were equilibrated overnight with 75 μCi $^{22}Na^+$ per ml. (A) The fraction of $^{22}Na^+$ remaining in reconstituted receptor vesicles (●———●), receptor-enriched membranes (○———○) and in the absence of receptor vesicles or membranes (▼———▼) was determined on upper GF/C disks. The fraction of reconstituted receptor vesicles remaining on the GF/C disks was estimated with the lipid tracer cholesteryl [1-^{14}C]oleate (□———□). (B) Same as in (A) except that serial washing was performed with upper DE81 disks.

tain an order of magnitude more intravesicular $^{22}Na^+$. Note also the rather stringent adherence of the reconstituted receptor vesicles (lipid tracer) to the upper GF/C disks throughout the wash and transfer cycles.

Similar serial washing profiles of $^{22}Na^+$ are also obtained with upper DE81 disks except that free tracer is less effectively removed with about 0.002 fraction remaining at the termination of the assay (Fig. 1B). In agreement with the results in Table I, there is 30% less retention of the reconstituted receptor vesicles on the upper DE81 disks as compared to the GF/C type. Since faster filtration rates are also obtainable with upper GF/C disks, we exclusively utilize this filter type for all subsequent experiments. In the interest of brevity and interpretation, we present tracer efflux and influx studies with the more extensively characterized receptor-enriched membranes [2,4,513]. Agonist-inducible tracer efflux results based on absorption filtration with the reconstituted receptor vesicles have recently been presented elsewhere [20].

$^{22}Na^+$ efflux with receptor-enriched membranes

As described above, serial washing of $^{22}Na^+$ equilibrated membranes results in the separation of extravesicular from intravesicular tracer. Following the removal of extravesicular $^{22}Na^+$, the addition of carbamylcholine can produce an easily detectable agonist-induced release of intravesicular tracer (Fig. 2A and B). We observe that three 15 μl wash and transfer cycles with carbamylcholine are necessary for the complete accelerated release of intravesicular $^{22}Na^+$ (Fig. 2B). The serial washing profile then plateaus to that observed in the absence of agonist indicating desensitization of the

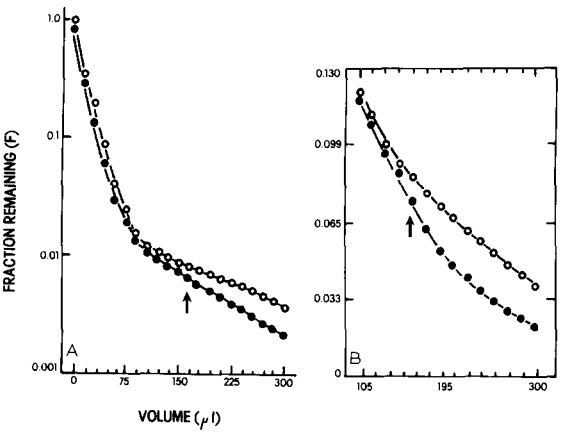

Fig. 2. (A) Serial washing diagram depicting the carbamylcholine-induced efflux of $^{22}Na^+$ from receptor-enriched membranes. Data are expressed as the fraction of $^{22}Na^+$ remaining on upper GF/C disks (presented on a log scale) versus each 15 μl wash and transfer cycle. The membranes were equilibrated overnight with 75 μCi $^{22}Na^+$ per ml. Following the removal of extravesicular tracer, ten additional 15 μl wash and transfer cycles were performed in the presence (●———●) and absence (○———○) of $1 \cdot 10^{-3}$ M carbamylcholine. Arrow denotes the first wash cycle with agonist. (B) Features of the same serial washing experiment are shown except that the ordinate is presented on a linear scale in order to illustrate the carbamylcholine-induced efflux of $^{22}Na^+$.

TABLE II

SODIUM EFFLUX WITH RECEPTOR-ENRICHED MEMBRANES

Carb, carbamylcholine; OcGlc, octylglucopyranoside.

	cpm $^{22}Na^+$			$\bar{F}$ [d]
	intravesicular [a]	Effluxed [b]	Retained [c]	
No Carb	2005 ± 135	454 ± 38	1551 ± 97	0.226 ± 0.004
Carb	2093 ± 77	955 ± 14	1138 ± 63	0.457 ± 0.01
Carb α-Bgt [e]	2046 ± 111	494 ± 22	1551 ± 88	0.242 ± 0.003
Carb Desens. [f]	1941	425	1516	0.220
Resens.	1889	739	1150	0.390
Gramicidin [g]	1290 ± 34	393 ± 22	887 ± 12	0.307 ± 0.009
Hypotonic [h]	2105 ± 40	1378	727 ± 60	0.655 ± 0.022
OcGlc [i]	2111 ± 78	1345 ± 172	766 ± 95	0.635 ± 0.058

[a] $^{22}Na^+$ remaining on upper GF/C disks after three 100 μl wash and transfer cycles. The membranes were equilibrated overnight with 75 μCi $^{22}Na^+$ per ml. Values are expressed as either single determinations or as averages of two or more determinations with respective standard deviations.

[b] $^{22}Na^+$ effluxed into underlying 934-AH filter with or without $0.5 \cdot 10^{-3}$ M carbamylcholine after wash cycles.

[c] $^{22}Na^+$ retained on upper GF/C disks after efflux step.

[d] $\bar{F}$ = cpm $^{22}Na^+$ effluxed/cpm $^{22}Na^+$ intravesicular.

[e] Membranes were preincubated with a 3-fold molar excess of α-bungarotoxin (α-Bgt) over α-toxin binding sites.

[f] Membranes were preincubated with $1 \cdot 10^{-5}$ M carbamylcholine for 30 min and the wash cycles were conducted either in the presence (Desens.) or absence (Resens.) of the same concentration of agonist.

[g] Membranes were preincubated for 30 min with 50 μg gramicidin per mg of membrane protein.

[h] After the wash cycles, the membranes were osmotically shocked with 10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 20 mM KCl, and 0.02% NaN_3.

[i] After the wash cycles, the membranes were washed with 15 mM octylglucopyranoside contained in membrane buffer (10 mM Tris-HCl, pH 7.4, 250 mM Nacl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.02% NaN_3).

membrane bound receptor. After analyzing these results, we economized routine efflux assays by removing the extravesicular $^{22}Na^+$ with three 100 μl wash cycles followed by a 50 μl efflux step with or without carbamylcholine.

Results from representative efflux experiments with native receptor-enriched membranes are shown in Table II. Following the removal of extravesicular $^{22}Na^+$, the fraction ($\bar{F}$) of tracer efflux in the presence of carbamylcholine (0.457 ± 0.01) is observed to be twice that in the absence of agonist (0.226 ± 0.004). The agonist-induced efflux is completely inhibited when the membrane-bound receptor is preincubated with a molar excess of α-bungarotoxin ($\bar{F}$ = 0.242 ± 0.003). The normalized value of $\bar{F}$ has proven to be a more reliable measure of tracer efflux efficiency than either the cpm $^{22}Na^+$ effluxed or retained. It is easy to calculate since absorption filtration allows the direct measurement of ^{22}Na effluxed from the intravesicular pool after the removal of extravesicular tracer. We have observed that the values of $\bar{F}$ are quite similar between numerous membrane preparations obtained under varying conditions and incubated with slightly different concentration of $^{22}Na^+$. Note that the values of cpm $^{22}Na^+$ retained, which are the only measurements routinely obtained by the vacuum filtration technique, generally exhibit standard deviations (±7.1%) twice as large as those associated with the value of $\bar{F}$ (±3.4%).

Tracer efflux by absorption filtration can also accommodate desensitization as well as resensitization experiments with the receptor-enriched membranes (Table II). Preincubation with carbamylcholine ($1 \cdot 10^{-5}$ M) desensitizes the membrane-bound receptor whereby the agonist induced efflux of $^{22}Na^+$ is completely inhibited ($\bar{F}$ = 0.220). Desensitization only occurs when the buffer used during the wash cycles also contains

the same concentration of carbamylcholine utilized during preincubation with the membranes. When agonist is excluded from the wash buffer, the membrane-bound receptor apparently becomes resensitized since the agonist-induced efflux is restored to a value reminiscent of activated acetylcholine receptor membranes ($\bar{F} = 0.390$). Under these conditions, the membrane-bound receptor can be effectively resensitized when $1 \cdot 10^{-4}$ M carbamylcholine or less is used for desensitization.

The effect of membrane perturbants on the tracer flux behavior of receptor-enriched membranes was also examined by absorption filtration (Table II). Preincubation with the ion-pore forming antibiotic gramicidin A induces apparent 'leakiness' in the receptor membranes since the amount of intravesicular $^{22}Na^+$ remaining after the wash cycles is considerably lower (1290 ± 34 cpm) and the efflux efficiency is higher ($\bar{F} = 0.307 \pm 0.009$) than in untreated membranes. When a hypotonic buffer or a buffer containing detergent is used during the efflux step, the value of $\bar{F}$ becomes greater than that usually observed in the presence of carbamylcholine with more than 50% of the intravesicular $^{22}Na^+$ effluxed. These results indicate that the deposition of receptor membranes on the GF/C disks does not alter the well-known effects of these membrane perturbations on tracer flux.

The well-characterized concentration effect of carbamylcholine and acetylcholine on the agonist-induced efflux of $^{22}Na^+$ from receptor-enriched membranes was also examined in order to verify the validity of the absorption filtration technique (Fig. 3). While the maximum agonist-induced tracer efflux is similar for both carbamylcholine ($\bar{F} = 0.457 \pm 0.01$) and acetylcholine ($\bar{F} = 0.471 \pm 0.01$), it is also concentration-dependent, occurring at $5 \cdot 10^{-4}$ M and $1 \cdot 10^{-4}$ M, respectively. The concentration of agonist producing 50% of the maximum response (C_{50}) occurs at $2.7 \cdot 10^{-5}$ M carbamylcholine and $0.9 \cdot 10^{-5}$ M acetylcholine. Lower concentrations of the natural neurotransmitter acetylcholine are sufficient to elicit the same efflux response observed with carbamylcholine.

Prolonged exposure of membrane-bound receptor to carbamylcholine causes desensitization which is also concentration-dependent (Fig. 3). The maximum agonist-induced efflux of $^{22}Na^+$ is reduced by 50% when the membranes are preincubated with $0.9 \cdot 10^{-6}$ M carbamylcholine and is completely inhibited when preincubated at $1 \cdot 10^{-5}$ M carbamylcholine.

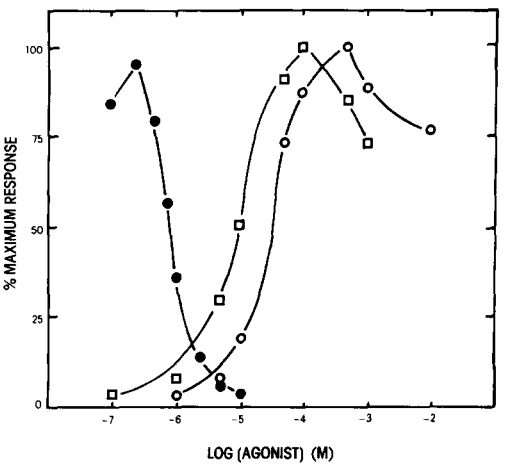

Fig. 3. The effect of varying the agonist concentration on the efflux of $^{22}Na^+$ from receptor-enriched membranes. Following overnight equilibration between tracer and membranes, extravesicular $^{22}Na^+$ was removed from the upper GF/C disks with three 10 μl wash and transfer cycles. Tracer efflux was performed with a 50 μl pulse of either carbamylcholine (○———○) or acetylcholine (□———□) at the indicated concentrations expressed on the abscissa. For desensitization studies, the membranes were preincubated for 30 min with the indicated concentrations of carbamylcholine (●———●). Extravesicular $^{22}Na^+$ was removed in the above manner except that the wash buffer contained the same conditioning concentration of agonist. Tracer efflux was then performed with a 50 μl test pulse of carbamylcholine ($0.5 \cdot 10^{-3}$ M).

$^{22}Na^+$ influx with receptor-enriched membranes

After depositing receptor membranes on the GF/C disks, tracer influx is performed with a 10 μl pulse of $^{22}Na^+$ with or without carbamylcholine. Serial washing profiles subsequent to the $^{22}Na^+$ test pulse again reveal the large exponential removal of extravesicular tracer during the early wash cycles (Fig. 4). In the absence of agonist the fraction of intravesicular $^{22}Na^+$ retained within the membranes ($\bar{F} = 0.0008$) approaches that observed with background levels ($\bar{F} = 0.0006$) obtained by depositing the pulse of tracer before the addition of membranes. Similar background levels are obtained whether receptor membranes or buffer

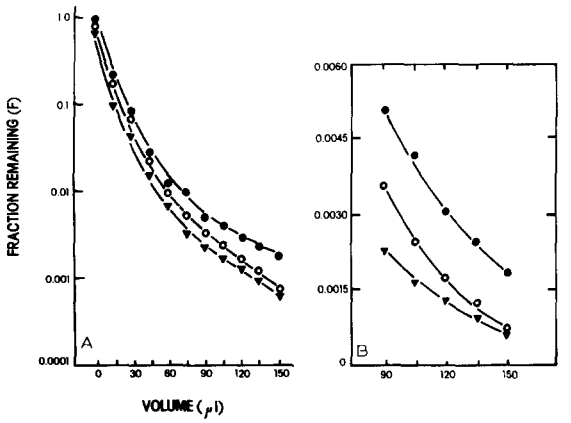

Fig. 1. (A) Serial washing diagram depicting the carbamylcholine-induced influx of $^{22}Na^+$ with receptor-enriched membranes. Data are expressed as the fraction of $^{22}Na^+$ remaining on upper GF/C disks (presented on a log scale) versus each 15 μl wash and transfer cycle. Tracer influx was initiated with a 10 μl pulse of $^{22}Na^+$ with (●———●) or without (○———○) $0.5 \cdot 10^{-3}$ M carbamylcholine. The serial washing profiles of free tracer (▼———▼) was obtained by depositing the membranes after the 10 μl pulse of $^{22}Na^+$. (B) Features of the same serial washing experiment are shown except that the ordinate is presented on a linear scale in order to illustrate the carbamylcholine-induced influx of $^{22}Na^+$.

is added subsequent to tracer. These latter findings appear to indicate that after absorption of the $^{22}Na^+$ the equilibration between tracer and membranes is quenched. Note also that the fraction of intravesicular $^{22}Na^+$ retained after rapid influx (occurring within one second) is less than 10% of that observed with membranes that are fully equilibrated overnight with tracer (Figs. 1A and 2).

In the presence of carbamylcholine there is approximately a 2-fold increase in the fraction of intravesicular $^{22}Na^+$ remaining ($\bar{F} = 0.0019$) after the ten 15 μl wash cycles (Fig. 4, A and B). For routine influx assays the serial washing procedure is substituted by three 100 μl wash cycles with representative results shown in Table III. After subtracting background levels of tracer, the amount of $^{22}Na^+$ influxed is observed to be ten times the amount in the absence of agonist. The agonist-induced influx of $^{22}Na^+$ is also concentration-dependent (Fig. 5) with a C_{50} value of $2.5 \cdot 10^{-5}$ M carbamylcholine which is similar to the value obtained in tracer efflux studies ($2.7 \cdot 10^{-5}$ M).

Desensitization of membrane-bound receptor by brief exposure to carbamylcholine can be easily

TABLE III

SODIUM INFLUX WITH RECEPTOR-ENRICHED MEMBRANES

Carb, carbamylcholine.

		cpm $^{22}Na^+$	
		retained [a]	influxed [b]
No Carb	Blank	409	
	Blank	368	
No Carb		491	103
		496	108
Carb		1396	1008
		1557	1167
Carb	Desens. [c]	461	73
	Desens.	542	154
[d] No Carb	Blank	513	
[d] No Carb		718	205
[d] Carb		2558	2045

[a] $^{22}Na^+$ retained on upper GF/C disks after three 100 μl wash cycles. Influx was initiated with a 10 μl pulse of $^{22}Na^+$ (75 μCi/ml) with or without $0.5 \cdot 10^{-3}$ M carbamylcholine to 10 μl of deposited membranes. For blank values, tracer is pulsed in the absence of membranes.

[b] $^{22}Na^+$ retained on upper GF/C disks after subtracting the average blank value.

[c] Membrane-bound receptor deposited on the upper GF/C disks was desensitized with a 10 μl pulse of $1 \cdot 10^{-4}$ M carbamylcholine before influx with $0.5 \cdot 10^{-3}$ M carbamylcholine containing $^{22}Na^+$.

[d] Following an initial influx assay, the entire assay was repeated on the same samples.

examined with the influx assay. A 10 μl conditioning dose of carbamylcholine ($1 \cdot 10^{-4}$ M) completely abolishes the $^{22}Na^+$ influx response induced by a subsequent test pulse of agonist containing tracer (Table III). Desensitization processes, as assessed by tracer influx, are also concentration-dependent with $2.5 \cdot 10^{-5}$ M carbamylcholine inhibiting 50% of the maximum response (Fig. 5). Due to the brief exposure, higher concentrations of carbamylcholine are necessary to elicit the same desensitization effect observed when membranes are preincubated for 30 min with the agonist (Fig. 3).

A particularly interesting feature of the influx assay is that the tracer uptake cycle can be repeated with complete fidelity on the original deposited membranes (Table III). A second pulse of carbamylcholine with tracer, subsequent to the removal of extravesicular $^{22}Na^+$ from the first

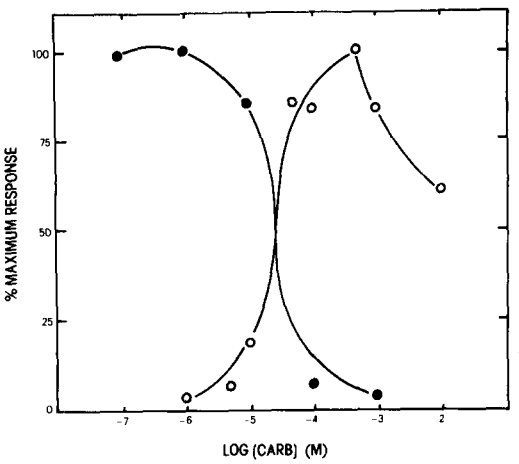

Fig. 5. The effect of varying the carbamylcholine concentration on the rapid influx of $^{22}Na^+$ with receptor-enriched membranes. The membranes were deposited on upper GF/C disks and tracer influx was initiated with a 10 μl pulse of $^{22}Na^+$ with the indicated concentrations of carbamylcholine expressed on the abscissa (○——○). Extravesicular tracer was removed by three 100 μl wash and transfer cycles. For desensitization studies, a 10 μl conditioning pulse of carbamylcholine at the indicated concentrations (●——●) was added to the membranes immediately before the test pulse of tracer containing $0.5 \cdot 10^{-3}$ M carbamylcholine.

influx cycle, doubles the amount of intravesicular $^{22}Na^+$ retained. Apparently, the original pulse of carbamylcholine is effectively removed along with the extravesicular $^{22}Na^+$ during the first 3 wash cycles, thereby resensitizing the membrane-bound receptor for the additional pulse of agonist. The results also indicate that the original intravesicular pool of $^{22}Na^+$ is stable throughout the six 100 μl wash and transfer cycles. Repetitive tracer influx is possible since the receptor-enriched membranes adhere tightly to the upper GF/C disks.

Discussion

An easy, inexpensive, rapid and reproducible method has been introduced to measure ion tracer flux. The absorption filtration technique exhibits comprehensive data retrieval whereby experimentally-induced error can be easily detected and accurately discarded from data analysis. Due to the vast array of available filter types, tracer flux studies with a variety of native membranes and reconstituted lipid vesicles systems of varying lipid composition and vesicular size can be accommodated. Absorption filtration should also prove to be a reliable method for determining vesicular volumes since the removal of extravesicular from intravesicular tracer can be accurately analyzed. An analogous technique has been successively utilized in examining the binding of amphiphatic compounds to a variety of cell membranes and synthetic phospholipid vesicles [8].

Although absorption filtration appears to be an attractive alternative to the more conventional approaches for measuring tracer flux, it does not approach the millisecond time domain of either rapid-mix quenched-flow [4,5] or stopped-flow fluorescence techniques [21]. On the other hand, these technically advanced approaches are not without apparent limitations. Rapid-mix quenched-flow techniques are useful only when ionophore activation as well as inactivation can be tightly regulated in the subsecond domain. Although providing a continuous spectrum, stopped-flow fluorescence is presently suitable only for measuring thallium ion flux. Absorption filtration may then serve as an important adjunct to the rapid kinetic approaches, since the establishment of ionophore activity should precede the expense of investigating rapid kinetics of ion-transport processes.

Native receptor-enriched membranes and electroplax lipid vesicles prepared with or without purified receptor protein adhere tightly to GF/C glass fiber disks. Following absorption onto the disks, extravesicular $^{22}Na^+$ can be separated from the intravesicular tracer retained with the deposited samples. These results mean that the signal-to-noise ratio is maximized in tracer efflux assays since activating ligands are applied to the deposited samples only after removal of extravesicular tracer. Thus a concentration-dependent carbamylcholine-induced efflux of $^{22}Na^+$ is easily detected in receptor-enriched membranes. Preincubation of membrane-bound receptor with conditioning doses of carbamylcholine also causes a concentration-dependent decrease in the efflux response to agonist tested after removal of extravesicular tracer. Desensitization is reversed when agonist is excluded from the buffer utilized for the wash and transfer cycles. Apparently, the conditioning dose of carbamylcholine is effectively removed whereby the agonist-induced efflux is restored. This feature

of absorption filtration is useful when the removal of activating or inactivating ligands in equilibrium with membrane ionophores is necessary before analyzing the permeability response elicited by the same or different ligand. For example, the receptor protein can be alkylated at or near the ligand binding site(s) with the affinity reagents maleimido-benzyltrimethylammonium iodide [13,22] or bromoacetylcholine [13,23–25]. Free and noncovalently-bound ligand must be removed by either dialysis or membrane sedimentation before the effect of specific alkylation on agonist-induced tracer flux can be examined. Fractionation procedures can become cumbersome especially when the membranes are reacted with varying concentrations of reagent in order to study the effect of fractional occupancy of the binding sites on the permeability response induced by agonist. This methodology problem can be circumvented entirely by the normal operational procedures of absorption filtration. In severe cases where unusually extensive washing may deplete intravesicular tracer, the efflux assay can be substituted with the influx approach.

Tracer influx is initiated with a brief 10 μl pulse of agonist plus $^{22}Na^+$ to receptor-enriched membranes previously deposited on GF/C disks. Following the removal of extravesicular tracer, a concentration-dependent carbamylcholine-induced influx of $^{22}Na^+$ is observed with a C_{50} value ($2.5 \cdot 10^{-5}$ M) similar to that observed by tracer efflux. Desensitization of membrane-bound receptor is also concentration-dependent whereby a brief pulse of conditioning carbamylcholine reduces the tracer influx response to agonist applied subsequently.

As mentioned above, free and noncovalently-bound ligands can be removed concomitantly with extravesicular tracer during wash and transfer cycles. This feature can be optimally exploited in the tracer influx assay. Following the removal of extravesicular tracer from an initial pulse of agonist and $^{22}Na^+$, a second pulse of carbamylcholine and tracer doubles the effective uptake of $^{22}Na^+$ in receptor-enriched membranes. Since the membranes adhere tightly to the GF/C disks and the accumulating pool of intravesicular $^{22}Na^+$ appears quite stable, agonist-induced tracer influx can likely be repeated numerous times. This may be particularly beneficial in those cases where tracer flux activity is not as discernable as in the native receptor-enriched membranes.

The absorption filtration technique was in fact especially designed to measure tracer flux in the reconstituted receptor vesicles. As shown above, these lipid vesicles retain an order of magnitude more intravesicular $^{22}Na^+$ than the native receptor-enriched membranes (Fig. 1). These observations can be partially ascribed to the fact that the receptor membranes are a consequence of subcellular fractionation whereby membrane fragments as well as sealed membrane structures are formed. On the other hand, the reconstituted receptor vesicles are produced from detergent/lipid/protein mixed micelles by the octylglucopyranoside-dialysis method which predominately forms sealed vesicles with average external diameters greater than 200 nm [10,26,27]. Note that lipid vesicles produced by sonication [28] or cholate-dialysis [27,29,30] invariably exhibit external diameters of 25–60 nm with internal volumes 1000-fold smaller than those obtained by octylglucopyranoside-dialysis [26]. Even though large internal volumes are advantageous for ion transport studies, conventional methodology for measuring tracer flux has been less than suitable in discriminating the flux signal from noise due to the larger intravesicular pools of tracer. With the advent of the absorption filtration technique, however, a concentration-dependent agonist-induced flux of $^{22}Na^+$ is clearly observable in the reconstituted receptor vesicles [20]. As a consequence of the large internal volumes and the repetitive nature of absorption filtration, the flux signal-to-noise ratio can be maximized beyond that obtainable with the native membranes. This will undoubtedly facilitate any study where amplification of the flux signal is necessary to ascertain subtle effects induced by various ligand-receptor interactions or alterations in the receptor microenvironment such as the lipid-receptor boundary.

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References

1 Conti-Tronconi, B.M. and Raftery, M.A. (1982) Annu. Rev. Biochem. 51, 491–530
2 Kasai, M. and Changeux, J.-P. (1971) J. Membrane Biol. 6, 1–23
3 Epstein, M. and Racker, E. (1978) J. Biol. Chem. 253, 6660–6662
4 Hess, G.P., Cash, D.J. and Aoshina, H. (1979) Nature (London) 282, 329–331
5 Neubig, R.R. and Cohen, J.B. (1980) Biochemistry 19, 2770–2779
6 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Suslinna, E.-M. and Racker, E. (1976) Anal. Biochem. 72, 57–65
7 Grunhagen, H.H. (1980) Anal. Biochem. 109, 18–26
8 Conrad, M.J. and Singer, S.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5202–5206
9 Thomas, C.A., Jr., Saigo, R., McLeod, E. and Ito, J. (1979) Anal. Biochem. 93, 158–166
10 Gonzalez-Ros, J.M., Paraschos, A. and Martinez-Carrion, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1796–1799
11 Clark, D.G., Mac Murchie, D.D., Elliot, E., Wolcott, R.G., Landed, A.M. and Raftery, M.A. (1972) Biochemistry 11, 1663–1668
12 Ong, D.E. and Brady, R.N. (1974) Biochemistry 13, 2822–2827
13 Delegeane, A.M. and McNamee, M.G. (1980) Biochemistry 19, 2822–2827
14 Gonzalez-Ros, J.M., Paraschos, A., Farach, M.C. and Martinez-Carrion, M. (1981) Biochim. Biophys. Acta 643, 407–420
15 Sator, V., Gonzalez-Ros, J.M., Calvo-Fernandez, P. and Martinez-Carrion, M. (1979) Biochemistry 18, 1200–1206
16 Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349–354
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Gonzalez-Ros, J.M., Llanillo, M., Paraschos, A. and Martinez-Carrion, M. (1982) Biochemistry 21, 3467–3474
19 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
20 Paraschos, A., Gonzalez-Ros, J.M. and Martinez-Carrion, M. (1982) Biochim. Biophys. Acta 691, 249–260
21 Moore, H.-P. and Raftery, M.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4509–4513
22 Weill, C.L., McNamee, M.G. and Karlin, A. (1974) Biochem. Biophys. Res. Commun. 61, 997–1003
23 Damle, V.W., McLaughlin, M. and Karlin, A. (1978) Biochem. Biophys. Res. Commun. 84, 845–851
24 Moore, H.-P.H. and Raftery, M.A. (1979) Biochemistry 18, 1862–1867
25 Wolosin, J.M., Lyddiatt, A., Dolly, J.O. and Barnard, E.A. (1980) Eur. J. Biochem. 109, 495–505
26 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840
27 Racker, E., Violand, B., O'Neal, S., Alfonzo, M. and Telford, J. (1979) Arch. Biochem. Biophys. 198, 470–477
28 Huang, C. (1969) Biochemistry 8, 344–351
29 Bruner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322–331
30 Wu, W.C.-S. and Raftery, M.A. (1981) Biochemistry 20, 69–701