PREPARATION OF SEALED *TORPEDO MARMORATA* MEMBRANE FRAGMENTS SUITABLE FOR QUANTITATIVE TRACER FLUX STUDIES

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1. Introduction

Sealed vesicular membrane fragments (microsacs) prepared from *Torpedo* electric organs have been used extensively in tracer flux experiments. Microsac suspensions with high AcChR and low AcChE content are obtained by density gradient separation of crude electric organ homogenates [1–5]. Special methods selectively yielding gradient fractions containing microsacs with functionally intact AcChR have been developed [6,7]. However, recent advances in the techniques [8–10] and in the mathematical analysis [11,12] of flux measurements, raise the need for more suitable methods of preparation.

The rate of tracer flux from or into a microsac is directly proportional to the number of receptor-controlled channels on the microsac, and inversely proportional to the microsac volume [12]. To facilitate quantitative analysis of flux data, suspensions containing microsacs with uniform size and receptor content are required.

Using the new Percoll density medium [13], a technique for preparing microsacs particularly suited for quantitative flux studies has been developed. Important novel features are:

(i) The use of the non-radioactive, and therefore non-hazardous, cation Li^+, which can be measured at sub-μM levels using FES;
(ii) Circumvention of receptor desensitization during selective neuroactivator-induced filling (AcCh pulses) of functional microsacs with Li^+;
(iii) Separation of Li^+-filled and Cs^+-filled microsacs using a continuous self-generating Percoll gradient;
(iv) Extensive characterization of the resulting fractions on the basis of stringent criteria.

Abbreviations: AcCh, acetylcholine; Carb, carbamoylcholine; d-TbC, d-tubocurarine; PMSF, phenylmethylsulfonylfluoride; AcChR, acetylcholine receptor; AcChE, acetylcholinesterase (EC 3.1.1.7); α-BuTx, α-bungarotoxin; Hepes, *N*-hydroxyethylenepiperazine-*N'*-ethylenesulfonic acid; FES, flame emission spectroscopy

2. Materials and methods

2.1. *Preparation of vesicular membrane fragments*

Microsacs were prepared from the electric organs of *Torpedo marmorata* (live from Station Biologique d'Arcachon) using a method based on that in [1]. The selective separation of functional and non-functional microsacs was adapted from the methods in [6,7]. Except where otherwise indicated, all procedures during the preparation were carried out at 4°C and the resuspension of pellet material was performed with a Potter homogenizer. Liquid N_2-frozen electric organ tissue (≈200 g) was broken up with a hammer and added, with stirring to ~400 ml preparation medium, containing 1 mM $CaCl_2$, 0.02% NaN_3 and 0.1 mM PMSF (Sigma), at 20°C. The suspension was homogenized in a Virtis 45 homogenizer with a double blade (near maximum speed) for 2 min and then centrifuged at 10 000 × *g* for 15 min. The supernatant fluid (S1) was retained, while the pellet was rehomogenized and centrifuged. The ensuing supernatant (S2) was combined with S1, filtered through cheese cloth and centrifuged at 22 000 × *g* for 2 h. The resulting pellet was resuspended in 30 ml solution containing 0.2 M CsCl, 1 mM $CaCl_2$, 5 mM Na-phosphate (pH 7.0). After 18 h incubation, the suspension was centrifuged at 22 000 × *g* for 30 min and the pellet washed with 45 ml flux medium containing: 0.2 M

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NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 5 mM Na-phosphate (pH 7.0). The microsac external CsCl level is thereby reduced to ~10 mM. This suspension was again centrifuged and the pellet taken up in 40 ml 0.2 M LiCl, 1 mM $CaCl_2$, 5 mM Na-phosphate (pH 7.0). Small aliquots of a 10 mM AcCh solution were added to the suspension under stirring, at 20 s intervals, transiently reaching 0.1 mM in AcCh after each addition. Due to the AcChE activity each aliquot was estimated to be hydrolyzed in ≈50 ms; final concentration of the hydrolysis product choline was ≈1 mM.

To reduce the microsac external Li^+ to 10^{-8} M, the suspension was washed twice with 150 ml flux medium and centrifuged. The pellet was resuspended in 35 ml flux medium and loaded onto Percoll (Pharmacia) density gradients (17% (v/v) Percoll in flux medium). The concentrated Percoll suspension was initially brought to pH 7.0 with solid Hepes buffer. Centrifugation (Beckmann L-50) at 15 000 × *g* for 30 min, yields 2 separate diffuse bands. The gradients were fractioned (LKB-Uvicord II) into 10 fractions of ~0.7 ml.

2.2. *Characterization of the Percoll gradient fractions*

The concentration of AcChR sites was determined using an ^{125}I-labelled (^{125}I-)α-BuTx (NEN Chem) binding assay in [14]. AcChE activity was determined by the pH-stat method [15]. Protein was estimated using the colorimetric method in [16]; the absorbance was read 2 min after mixing of dye and protein samples to avoid interference from Percoll [17]. The Li^+ concentration in the fractions was measured at 670 nm by FES (Unicam SP1900).

2.3. *Measurement of Li^+ efflux from microsacs*

The flux experiments were carried out with Percoll density gradient fractions of maximum AcChR specific activity, using a Sartorius multiple suction apparatus, modified so that suction is controlled by electrically triggered magnetic valves, coupled to digital clocks. The flow rate of liquid through the filter was adjusted to 1.7 ml/s; 0.3 ml microsac suspension were added to an ice-cooled bath under stirring. Aliquots (5 ml) of the suspension were then filtered, and the filters washed with 5 ml portions of ice-cold wash medium.

The Li^+ content of the filters added to 5 ml 1% Triton X-100 solution in a counting vial was measured by FES after shaking the vials for 17 h.

3. Results and discussion

The distribution of AcChR content and specific activity, AcChE activity, and total protein content for various fractions of a continuous Percoll gradient is shown in fig.1. As is also consistently found using sucrose gradients [1–4], there is a clear separation of AcChR-rich, and AcChE-rich fractions. An important step in the procedure of preparation is the selective, AcCh-induced uptake of LiCl into microsacs having functional receptors. The non-functional microsacs are expected to remain filled with CsCl. Two parallel runs were carried out for comparison, under conditions where:

(i) A corresponding amount of buffer was added instead of AcCh;

(ii) The microsacs were incubated in 3 × 10^{-6} M d-TbC for 5 min, then AcCh was added as before.

The resulting Li^+ content of the various fractions for each type of run is shown in fig.2. When buffer is added instead of AcCh there is some uptake of Li^+, presumably due to leakage influx during the incubation period. The Li^+ uptake is greatly increased when induced by AcCh pulses. A comparison with the data shown in fig.1 indicates that the Li^+ content of the fractions then closely parallels the content of toxin-binding sites. This is expected since the repeated addition of AcCh aliquots should ultimately lead to a fill-

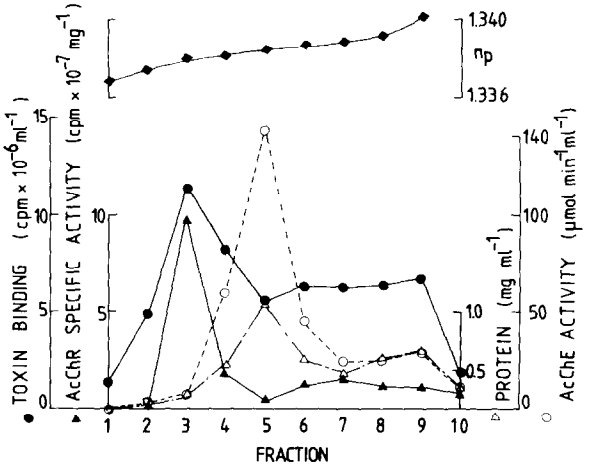

Fig.1. Distribution of ^{125}I-αBuTx, AcChE activity, AcChR specific activity, and total protein of *Torpedo marmorata* microsacs in a Percoll gradient. The density of Percoll (upper trace) in the individual fractions is measured in terms of the refractive index n_p.

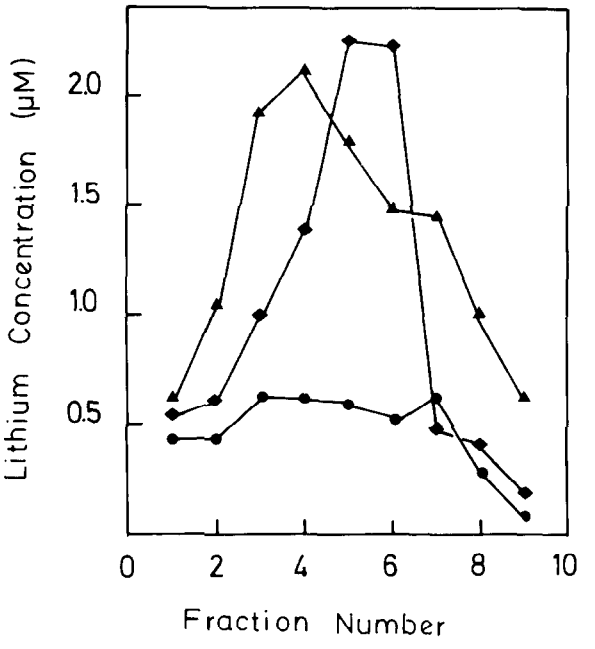

Fig.2. Distribution of Li^+ content in 3 separate Percoll gradients generated under identical conditions, using the same crude homogenate of *Torpedo marmorata* microsacs: (a) repeated addition of AcCh to induce Li^+ (–▲–); (b) a corresponding amount of flux medium was added instead of AcCh (–●–); (c) microsacs first incubated in d-TbC (3 μM) for 5 min, before addition of AcCh as in (a) (–◆–).

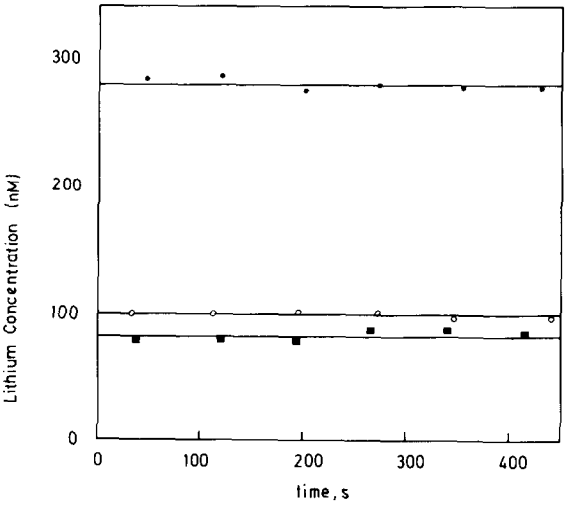

Fig.3. Efflux of Li^+ from *Torpedo marmorata* microsacs; Li^+ content determined by filtration of microsacs (fraction 3 of a Percoll gradient) at time *t* after initial dilution into a bath: (a) dilution into flux medium (–●–) and into Carb (0.5 mM) (–○–), respectively – filters washed with 4 × 5 ml flux medium; (b) dilution into flux medium followed by filtration and washing by successively 5 ml AcCh (0.1 mM), 5 ml flux medium, 5 ml AcCh (0.1 mM) and 5 ml flux medium (–■–). This leads to a complete emptying of Li^+ content (J. B., E. N., unpublished).

ing with Li^+ of all microsacs containing at least one functional receptor. The advantage of using AcCh instead of other neuro-activators (e.g., Carb) to induce Li^+ uptake is that rapid removal of AcCh by AcChE activity prevents extensive desensitization of receptors. As is shown in fig.3, due to desensitization, even high concentrations of Carb will not induce a complete efflux of Li^+ from microsacs filled with LiCl.

The dissociation equilibrium constant for the binding of d-TbC to AcChR, is 0.34 μM [18]. Preincubation of the microsacs with 3 μM d-TbC should therefore lead to inhibition of a large fraction of the receptors. After the subsequent addition of AcCh there will be a competition of AcCh and d-TbC for receptor binding sites. However, since the dissociation time constant for d-TbC is on the order of 10–100 ms [18], degradation of AcCh by AcChE is expected to successfully compete with displacement of d-TbC by AcCh. During its transient period of action the AcCh will therefore primarily bind to the small residual fraction of receptors not inhibited by d-TbC. Considerations based on the statistics of ligand binding to equivalent and independent sites on the microsacs [11,12] dictate that it is more probable that a microsac with many receptors will have residual receptors uninhibited by d-TbC than one with fewer receptors. For uptake of Li^+ induced by AcCh in the presence of d-TbC it is therefore expected that microsacs with a higher receptor content are selectively filled with LiCl. Comparison of the data in fig.2 indicates that a narrower distribution of Li^+ content over the gradient fractions results when d-TbC is present during AcCh addition. The Li^+ content of fractions 1–4 and 7–9 is then markedly decreased. The arguments presented above suggest that these fractions therefore contain microsacs with fewer functional receptors/microsac, than the microsacs in fraction 5 and 6.

The volume distribution of the microsacs in a given fraction was estimated using Sartorius membrane filters of pore sizes 0.45 μm and 0.8 μm. The filters will retain all microsacs with a diameter larger than the pore size. The fraction of the microsacs added to the filter that will be retained by filters of pore size

Table 1

Filter pore size (μm)	Fraction		
	3	5	7
0.45	60.6	45.5	43.4
	43.2[a]	42.2[a]	35.0[a]
0.8	19.6	19.6	24.6
	14.3[a]	19.0[a]	16.6[a]

[a] AcCh-activated uptake of Li^+ in the presence of 3×10^{-6} M d-TbC

The percent of Li^+ lithium retained by the filters of various pore size, for 3 separate microsac fractions in the Percoll gradient

0.45 μm should thus be greater than or equal the fraction retained by filters of pore size 0.8 μm. The qualitative trends in the percentage Li^+ retained, for filtrations using fractions 3,5 and 7, can be determined from the data given in table 1. The largest percentage of Li^+ in microsacs with a diameter bigger than 0.45 μm and 0.8 μm is contained in fractions 3 and 7, respectively. This suggests that, with increasing fraction number, there is an increase in the average size of the microsacs in a given fraction. When the AcCh-induced uptake of Li^+ is carried out in the presence of 3 μM d-TbC, the percentage Li^+ retained for fraction 3 and 7 is remarkedly reduced, for both types of filter. One may infer that for these two fractions the average number of receptors/microsac is lower than for fraction 5.

The procedure presented above was found to be highly reproducible. Fraction 3, having the highest AcChR content, a low AcChE content, a low average number of functional receptors, and a relatively small average microsac volume was judged to be optimum for flux studies. As shown in fig.3, >70% of the Li^+ is contained in functional microsacs. An alternative choice would be fraction 7, which has a higher AcChE content, but also a larger average microsac volume.

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