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ACTIVE Ca^{2+} TRANSPORT BY VESICLES RECONSTITUTED FROM TRITON X-100-SOLUBILIZED PIGEON ERYTHROCYTE MEMBRANE

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Summary

Pigeon erythrocyte membrane was solubilized partially, but relatively unselectively by Triton X-100. Vesicles were reconstituted from mixtures of Triton-solubilized membrane and lipid (phosphatidylcholine plus phosphatidylethanolamine plus cholesterol) by addition of bovine high-density lipoprotein. This efficiently removed the Triton X-100. Sodium dodecyl sulfate-polyacrylamide gel electropherograms of reconstituted vesicles showed band patterns resembling those of the original membrane. The reconstituted vesicles showed ATP-dependent active accumulation of $^{45}Ca^{2+}$. ATP-dependent $^{45}Ca^{2+}$ uptake by the reconstituted vesicles resembled the corresponding activity of the original membrane vesicles; in both preparations the Ca^{2+} uptake rate depended on the square of the Ca^{2+} concentration and had similar $[Ca^{2+}]_{1/2}$ values, 0.16 μM and 0.18 μM, respectively.

Introduction

Several procedures are available for reconstituting transport-active vesicles from detergent-solubilized membranes; for example, Refs. 1—9. No procedure is quick, gentle and efficient in removing the detergent. An ideal general procedure should also be unselective for both solubilization and reconstitution.

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Abbreviations: SDS, sodium dodecyl sulfate; HDL, bovine high-density lipoprotein, d = 1.17, prepared and density calculated as described in Ref. 10 and footnote p. ■■; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

We observed that high-density lipoprotein(HDL) quickly and efficiently abstracts Triton X-100 from pigeon erythrocyte membranes at 0°C [10]. This suggested that HDL might be useful for reconstitution of Triton-solubilized membrane. In the experiments described below, membrane was solubilized at 0°C with Triton X-100 and transport-active vesicles formed by adding lipid and HDL. Both membrane solubilization and reconstitution appear relatively non-selective as judged by SDS-polyacrylamide gel electrophoresis. The reconstituted vesicles possess an ATP-dependent active Ca^{2+} transport activity which is very similar to the corresponding activity of the original membrane vesicles.

Materials and Methods

[*Methyl*-^{3}H]Choline chloride was from Amersham/Searle (Arlington Heights, IL), [*phenyl*-^{3}H(N)]Triton X-100 and ^{22}NaCl were from New England Nuclear (Boston, MA), and $^{45}CaCl_2$ was from ICN (Irvine, CA). The scintillation cocktail (3a70B) was from Research Products International Inc. (Elk Grove Village, IL). L-α-Phosphatidylcholine (Type III-E, from egg yolk), L-α-phosphatidylethanolamine (Type III, from egg yolk), cholesterol (CH-K, Ash free), Triton X-100 and Sephadex G-50-150 were from Sigma Chemical Co. (St. Louis, MO). Asolectin (soybean phospholipids) was from Associated Concentrates (Long Island, NY). Bio-Gel A-15m (100—200 mesh) was from Bio-Rad Labs. (Richmond, CA). Acrylamide and *N,N'*-methylenebisacrylamide were from Aldrich Chemical Co. (Milwaukee, WI). Inorganic chemicals were reagent grade or better. Water was double deionized and all glassware was rinsed with double-deionized water. Bovine high-density lipoprotein was prepared by a modification * of the procedure described in Ref. 10.

Preparation of membrane extract. Pigeon erythrocyte membrane vesicles were prepared as described previously [11] with slight modifications: membranes were in KCl/phosphate (no EGTA) during the second sonication, which was done at setting 5 for three times 10-s periods alternated with 60-s cooling intervals (0°C) on 4-ml portions in Corex tubes. Membrane was extracted with Triton X-100 at 0°C for 30 min in a solution that contained 100 mg membrane (wet weight) and 6 μl Triton X-100/ml, 135 mM KCl, 5 mM dithiothreitol, 18.3 mM Tris-HCl (titrated to pH 8.3 at 0°C as a five-times concentrated stock) and 0.2 mM KN_3. The mixture was centrifuged for 45 min at 2—4°C and 45 000 rev./min in a Type 65 fixed angle rotor in a Beckman L5-65 ultracentrifuge. The supernatant was held at 0°C and was used as soon as possible for reconstitution. (Very approximately, half the transport activity could be reconstituted after holding the extract 8 h at 0°C.)

Preparation of lipid dispersions. Lipid mixtures in $CHCl_3$ (30 mg lipid/tube)

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* First, some misprints in Ref. 10 escaped our notice. The first centrifugation was at 6000 × *g*, not 600 × *g*, the supernatant was brought to 0.55% Dextran sulfate, not 1.55% and the material held overnight at 0—4°C was brought to 16% ... with 0.02 M, not 0.02% Tris-HCl. The modifications of the procedure were as follows. (1) After the addition of Tris-HCl (above), the solution was centrifuged 10 min, 15 000 × *g* at room temperature and the resulting white pellet discarded before bringing to 0.2 M in $CaCl_2$ and centrifuging to recover the precipitated yellow-orange HDL. The pellet was dissolved in citrate and sequentially precipitated with Tris-HCl and $CaCl_2$ twice more. (2) The final dialysis was done twice instead of once.

were dried overnight with an N_2 stream and dispersed in 1.5 ml 126.8 mM KCl, 14.4 mM K_2HPO_4, 5.6 mM KH_2PO_4 2.5 μl/ml Triton X-100 by sonicating for 7.5 min at setting 3 in a 20°C bath. The resulting clear dispersion was centrifuged for 2 min at 5000 rev./min in an SS-34 head. The lipid mixture used for the experiments shown was egg yolk phosphatidylcholine/egg yolk phosphatidylethanolamine/cholesterol (2 : 2 : 1, w/w). Mixtures with asolectin replacing phosphatidylethanolamine work at least as well.

Reconstitution of vesicles. All operations were performed at 0°C unless otherwise stated. To 0.75 ml membrane extract were added 0.050 ml 75 mM $MgCl_2$, 0.050 ml 30 mM mercaptoethanol and a 'trapped space' marker ([^{3}H]-choline or $^{22}Na^+$). Then 0.25 ml lipid dispersion was added, the mixture was left for 20 min, 0.050 ml potassium phosphate 'neutralization' buffer added (138 mM KH_2PO_4, 162 mM K_2HPO_4, for Fig. 3 experiments; 106.5 mM KH_2PO_4, 43.5 mM K_2HPO_4 for the other experiments) and the mixture was left to stand for 10 min. 2.5 mg HDL was then added (as approx. 100 mg/ml solution in the final dialysis buffer of Ref. 10) and, 5 min later, another 12.75 mg HDL was added. After 20 min, the volume was brought to 1.50 ml with 154 mM KCl and the mixture kept at 39°C for 20 min and then at 0°C for 20 min. For all but the Bio-Gel filtration experiments (Figs. 1 and 2), the reconstituted vesicles were separated from extravesicular low molecular weight components by passage through Sephadex G-50 columns as described earlier [12]. The columns were equilibrated and eluted with 123 mM KCl, 2.5 mM $MgCl_2$ 14.4 mM K_2HPO_4, 5.6 mM KH_2PO_4. From each such 'preparative' column, a 6 ml vesicle fraction was collected.

Assay for ATP-dependent Ca^{2+} transport. $^{45}Ca^{2+}$ was added to two 1.0 ml aliquots of vesicle suspension from a preparative column and MgATP ($MgCl_2$ + Na_2ATP) was added to one aliquot. The final volume was 1.50 or 1.30 ml and final concentrations were: 2.5 or 0 mM MgATP, 5.0 mM NaCl, 2.5 mM $MgCl_2$, 5.6 mM KH_2PO_4, 14.4 mM K_2HPO_4, 'carrier-free' $^{45}CaCl_2$ or Ca^{2+} buffer (below); KCl to 304 mOsM. In some experiments ('trace' Ca^{2+}) only $^{45}Ca^{2+}$ was added, in others a Ca^{2+} buffer was used which was a mixture of CaEGTA, MgEGTA (total EGTA, 1 mM) plus $^{45}Ca^{2+}$.

The samples were incubated for the indicated times and temperatures (figure legends), chilled and analysed for vesicle-trapped $^{45}Ca^{2+}$ and [^{3}H]choline (the trapped space marker) by passage through Sephadex columns [12], mixing 4 ml from the 6-ml vesicle fractions with 10 ml 3a70B counting cocktail and counting in a Packard Tri-Carb scintillation counter. Typically, the Triton extraction of membrane through the collection of vesicle fractions from the analytical columns took 4 h.

For the Bio-Gel column experiments (Figs. 1 and 2), reconstitution was performed as described above except that [^{3}H]Triton X-100 was present in the added lipid and ^{22}NaCl was the trapped space marker instead of [^{3}H]choline. The first passage through Sephadex column was omitted to avoid dilution. Instead, 0.50 ml $^{45}Ca^{2+}$, 5.6 mM KH_2PO_4, 14.4 mM K_2HPO_4, 10 mM $MgCl_2$, ±10 mM MgATP and KCl to 304 mOsM was added to 1.5-ml reconstituted vesicles. The mixtures were incubated for 15 min at 39°C, chilled at 0°C and 1.5 ml was passed through Bio-Gel A-15m columns at 2—4°C to separate vesicles from the other components. Bio-Gel columns were equilibrated and

eluted with the same solution as the Sephadex columns. 5-min fractions (about 1.5 ml) were collected, of which 0.3 ml was counted for radioactivity, 0.2 ml was used for protein determination [13] and the rest precipitated for electrophoresis.

Preparation of protein of Bio-Gel fractions for SDS-polyacrylamide gel electrophoresis. Selected column fractions were pooled and precipitated with an equal volume of 100% (w/v) trichloroacetic acid/CH_3OH (1 : 9, v/v) at room temperature for 30 min, then centrifuged for 30 min in an Adams Sero-fuge or until the supernatant was clear. The pellet was washed twice with acetone. The remaining acetone was removed with a stream of nitrogen. The pellets were dispersed in 10—20 μl 154 mM NaCl, an equal volume of buffer (2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 117 mM glycine, 15 mM Tris base) added and the samples heated 5 min in a boiling water bath. Triton X-100 membrane extracts were prepared for electrophoresis the same way.

SDS gel electrophoresis. Electrophoresis was carried out in a Model 113 Acrylamide Gel Accessory for the Beckman Microzone System, using the recommended Tris/glycine buffer for reservoir and separating gel solutions [14]. In addition, a stacking gel (3.2 X 6.25) * was introduced, using 1/5 of the buffer concentration of the separating gel (7.5 X 5) *. All solutions were 1% in SDS. Sucrose was added to samples as a density agent and 15—100 μg protein/channel was applied to the gel. The gel was stained overnight in 0.5% Coomassie Blue R-250, in 7% acetic acid, 40% ethanol, and destained by agitated diffusion in 7% acetic acid and 20% ethanol for about 1 day.

Results

The extraction procedure releases 30.0 ± 15.5% (n = 8) of the membrane protein into the 130 300 X g_{av}, 45 min supernatant. Although membrane solubilization is incomplete, it appears relatively non-selective, as does reconstitution. The SDS gel electrophoresis patterns are similar for original membrane (Fig. 1, M), membrane extract ** (Fig. 1, E) and reconstituted vesicles (Fig. 1, V) except for the pair of high molecular weight bands possibly analogous to the spectrin bands of human erythrocytes.

Reconstituted vesicles were separated from the other components of the reconstitution mixture by chromatography on Bio-Gel A-15m ($1.5 \cdot 10^7$ dalton exclusion limit). The elution profiles of trapped space ($^{22}Na^+$) and ATP-dependent $^{45}Ca^{2+}$ uptake are shown in Fig. 2a and total protein and [^{3}H]Triton are shown in Fig. 2b. Trapped space, $^{45}Ca^{2+}$ uptake activity and a protein peak all coincided just behind the excluded volume. (Most of the protein in fractions 7—17 is HDL.) A small residual Triton peak was seen lagging the other peaks. The Triton/protein ratio (ml/g) in the vesicle peak was 0.028 ± 0.006 (n = 9, five experiments). This is about the minimum inhibitory Triton dose for

* Gel notation of Hjerten [16].

** Triton extracts of membrane invariably showed a strong band of unknown nature in the very low molecular weight region of SDS gel. We could not mimic this with lipid, Triton or lipid/Triton mixtures. Inclusion of 4.9 T.I. units/ml aprotonin (Sigma) in the Triton extraction solution and holding the extract 30 min at 0°C had absolutely no effect on the SDS gel patterns, nor did holding extracts without protease inhibitor at 0°C for 5—6 h before electrophoresis.

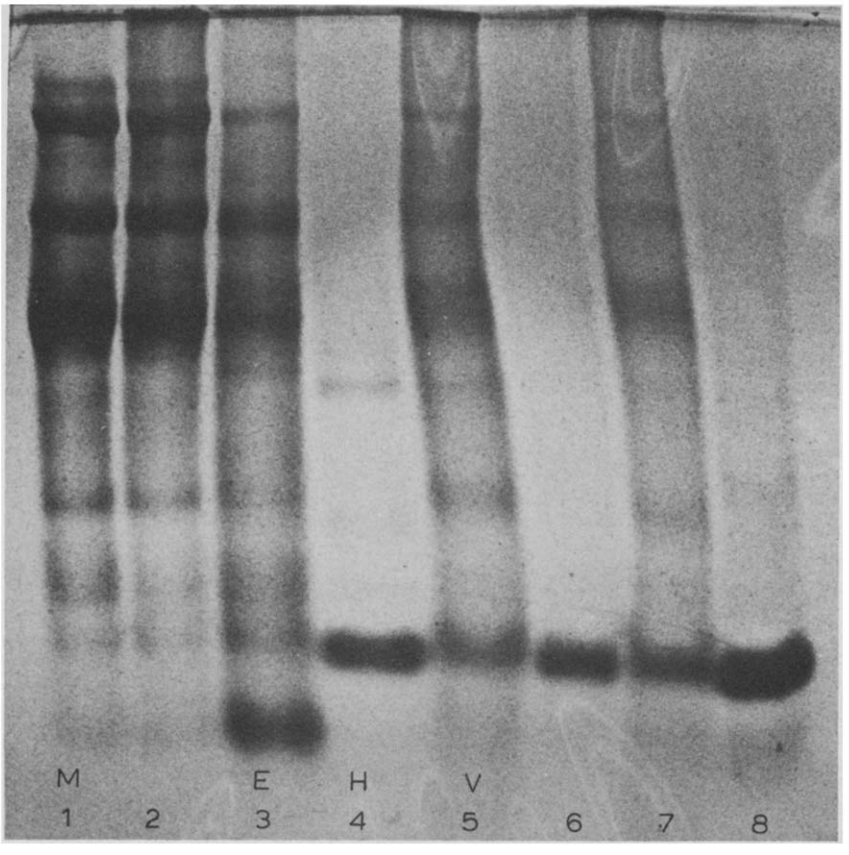

Fig. 1. SDS gel electrophoresis of membrane, extract and reconstituted vesicles. Channel M: membrane (not precipitated), 32 μg protein; channel E: precipitated Triton extract of membrane, 48 μg; channel V: precipitated reconstituted vesicle fractions 3 + 4 + 5 of Fig. 2 from the '+ATP' Bio-Gel column, 30 μg; channel H: precipitated HDL, 12 μg. Also run on this gel were precipitated membrane (32 μg; channel 2); column fraction 10, precipitated, from the '+ATP' and '—ATP' columns (14 μg and 18 μg, channels 6 and 8); and vesicle fractions, analogous to V, but from the '—ATP' column (42 μg, channel 7).

glycine transport [10], but much of this Triton should be associated with residual HDL (see Discussion). The input ratios of Triton to membrane protein and Triton to membrane protein plus HDL protein were 6.0 and 0.67, respectively. In the vesicle peak, the total protein (membrane protein plus HDL) was 37.8 ± 11.6% (n = 5, three experiments) of the input membrane protein. Most of the protein in the vesicle peak was membrane protein although some HDL was seen (Fig. 1, V and H).

In the remaining experiments to be described, activities in reconstituted mixtures were measured without separating vesicles from the other macromolecular components.

There were some specific lipid requirements for formation of transport-active vesicles (data not shown). With no added lipid there was no vesicle-trapped space. Vesicles formed from egg yolk phosphatidylcholine -cholesterol plus extract (trapped space appeared) but they had no ATP-dependent Ca^{2+}

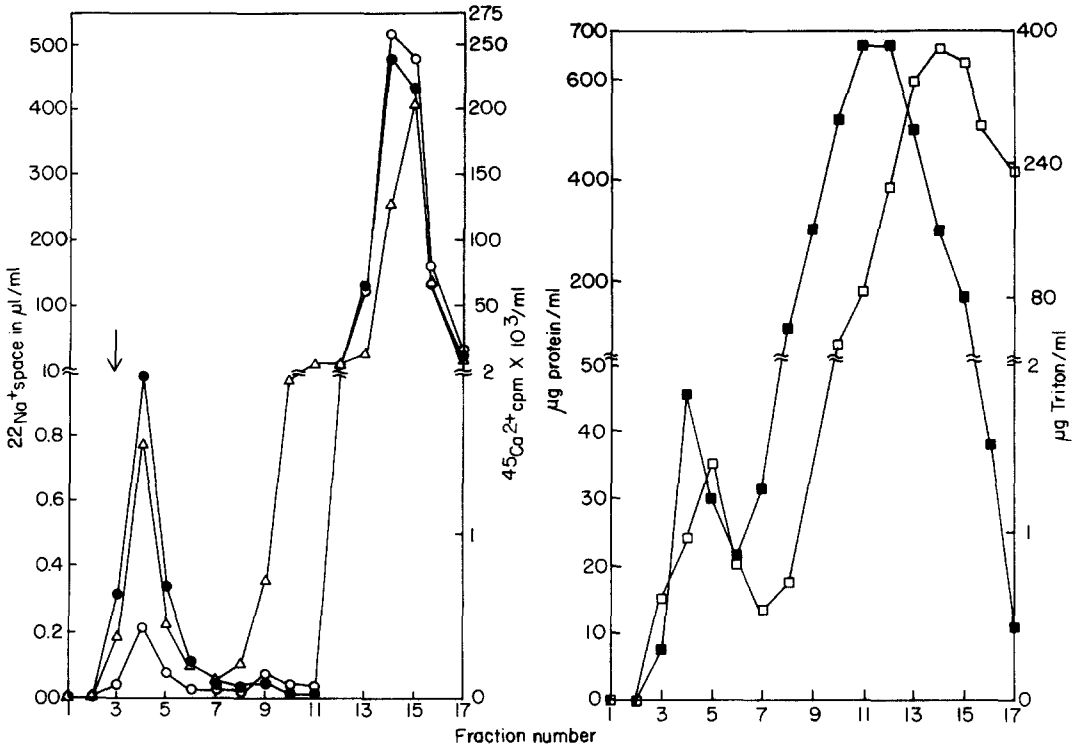

Fig. 2. Elution profiles from Bio-Gel columns of trapped space ($^{22}Na^+$) and $^{45}Ca^{2+}$ uptake (a) and protein and [^{3}H]Triton X-100 (b). There was 6 μCi ^{3}H/ml Triton in the lipid, 0.33 μCi $^{22}Na^+$/ml in the reconstitution mixture and 0.25 μCi/ml $^{45}Ca^{2+}$ in the uptake medium. $^{45}Ca^{2+}$ uptake was from 'trace' Ca^{2+} medium for 15 min at 39°C. (a) ●, $^{45}Ca^{2+}$, +ATP; ○, $^{45}Ca^{2+}$, —ATP; △, $^{22}Na^+$; arrow, the elution position of erythrocytes (excluded volume) determined on separate passage through columns. (b) ■, protein; □, Triton.

transport activity. Mixtures (2 : 2 : 1, w/w/w) of asolectin, phosphatidylcholine and cholesterol or mixtures of egg yolk phosphatidylethanolamine, phosphatidylcholine and cholesterol (2 : 2 : 1) gave active vesicles. (Knowles et al. [15] have reported the phosphatidylcholine alone will not support Ca^{2+} transport by a reconstituted sarcoplasmic reticulum system but phosphatidylcholine/phosphatidylethanolamine mixtures will.) Omission of cholesterol gave vesicles with less Ca^{2+} transport activity. Since HDL contains cholesterol, we did not attempt to determine if the cholesterol requirement was absolute.

Time and ATP-dependent $^{45}Ca^{2+}$ uptake are shown in Fig. 3. This uptake represents active accumulation since accumulated $^{45}Ca^{2+}$ was released * by the

* A referee asked for a plausible explanation for the observation that addition of the ionophore at zero time increased the $^{45}Ca^{2+}$ associated with the vesicles to a value above the equilibrium value of the ATP-free samples and above the value from the ATP sample treated later with the ionophore. Most of the Ca^{2+} associated with the vesicles is presumably bound to membrane components although in equilibrium with free Ca^{2+}. The amount of Ca^{2+}/ml enclosed space is far too high to represent only free Ca^{2+}. We presume a change in the 'equilibrium' value reflects a change in binding. This might arise from a drop in a Donnan ratio. Acidic groups (e.g. sialic acid) at the inner membrane face would produce a Donnan effect. Given a high surface/volume ratio, this could be appreciable. If vesicles swelled during incubation, the Donnan ratio ('f') would drop, producing a drop in $[Ca^{2+}]_i/[Ca^{2+}]_0$ proportional to f^2. Another possibility is that some non-vesicular material aggregates during incubation. It, and Ca^{2+} bound to it, would not pass through the Sephadex columns.

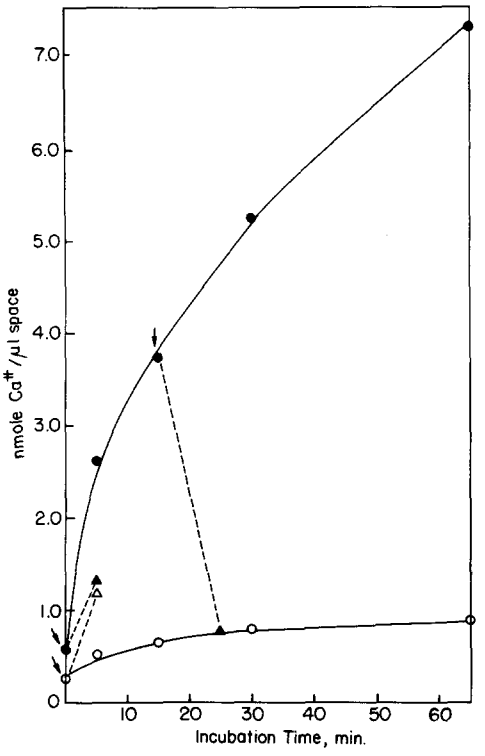

Fig. 3. Time and ATP-dependent $^{45}Ca^{2+}$ uptake by reconstituted vesicles. There was 8 µCi/ml [^{3}H]-choline (1 mM) in the reconstitution mixture and 2.2 µCi/ml $^{45}Ca^{2+}$ in the incubation medium together with 225 µM CaEGTA and 75 µM MgEGTA to serve as a Ca^{2+} buffer ([Ca^{2+}] = 0.81 µM). Samples were put into a 39°C bath at intervals and all samples removed and chilled in an ice bath at 65 min. The zero-time sample was left at 0°C. ●, ▲, ATP present; ○, △, ATP absent. - - - - - -△, - - - - - -▲, the effects of addition of A23187 at the times indicated by the left-hand termini (arrows) of the dashed lines. The final concentration of A23187 was 3 µM and its addition contributed 0.011% dimethyl formamide and 0.032% ethanol to the incubation medium.

Ca^{2+} ionophore, A23187. The Ca^{2+} uptake process of the reconstituted system corresponds to that of the original membrane vesicles. In both, the initial rate of ATP-dependent $^{45}Ca^{2+}$ uptake depends on the square of the Ca^{2+} concentration and $[Ca^{2+}]_{1/2}$ is similar for the native membrane vesicles (0.18 µM, Ting, A., Lee, J.W. and Vidaver, G.A., unpublished data) and reconstituted vesicles (0.16 µM, Fig. 4). The specific activities are more difficult to compare. However, assuming that 25% (i.e. 2/3 X 37.8%) of the added membrane protein appears in reconstituted vesicles (experiments of Fig. 2), the V value (pmol · min^{-1} · mg^{-1} protein, experiments of Fig. 4) for reconstituted vesicles was 69 and 90 (two experiments). For the original membrane, V was 1240 and 557 pmol · min^{-1} · mg^{-1} protein (two experiments, Ting, A., Lee, J.W. and Vidaver, G.A., unpublished data). However, increasing the lipid/membrane extract ratio increases total trapped space and total ATP-dependent $^{45}Ca^{2+}$ uptake disproportionately (Fig. 5a and b). Approx. 2.5 times more uptake/mg added protein was obtained with only modest reduction in the +ATP/—ATP uptake ratio.

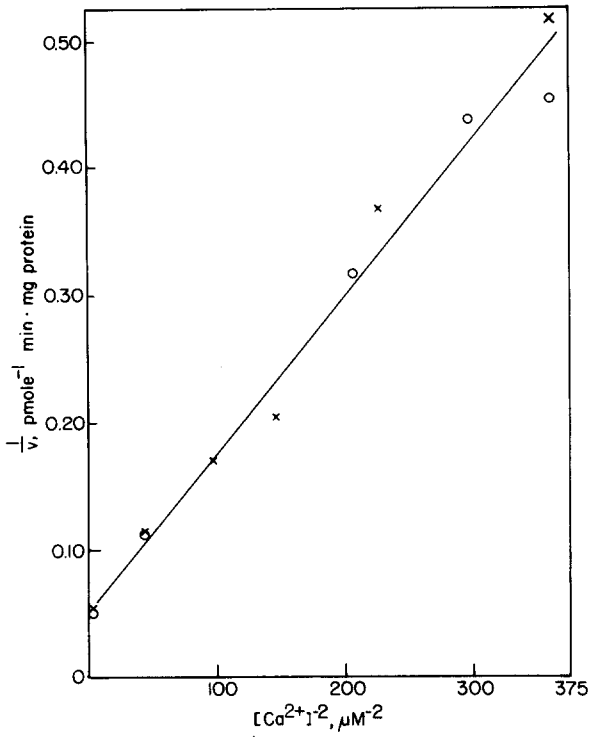

Fig. 4. Double-reciprocal plot of ATP-dependent $^{45}Ca^{2+}$ uptake vs. $[Ca^{2+}]^2$. $^{45}Ca^{2+}$ uptake was at 27°C for 35 min. $^{45}Ca^{2+}$ = 12 μCi/ml. Different $[Ca^{2+}]$ were obtained with EGTA buffers (total EGTA = 1 mM). Free $[Ca^{2+}]$ in each sample was calculated (Vidaver, G.A. and Ting, A., unpublished data). v is in pmol · min^{-1} · mg^{-1} total membrane protein in the reconstitution mixture. Data from two experiments were normalized to their average v for presentation in the same graph.

Discussion

One aim of this work was to devise a reconstitution procedure suitable for assaying transport components in membrane fractions. The procedure described is a step in this direction. It is quick and easy and a number of samples can be reconstituted and assayed in one working day.

The reconstituted vesicles resemble the original membrane in their SDS electrophoretic band pattern and Ca^{2+} transport kinetics.

Triton removal by HDL is nearly complete. The Triton/total protein ratio in the reconstituted vesicle fraction (0.028 ml/g) should be effectively below the minimum inhibitory dose (0.025 ml bound/g protein) found for glycine transport and vesicle sealing [10]. Much of the vesicle-associated Triton is presumably associated with the HDL in the vesicle fraction. If HDL is approx. 25—33% of the total reconstituted vesicle protein, the HDL protein/Triton weight ratio would be approx. 10. HDL will protect pigeon erythrocyte membrane from Triton damage at an HDL protein/Triton ratio less than 1 [10].

The low recovery of membrane protein in reformed vesicles and the low activity/mg vesicle protein have already been mentioned. This might be due to HDL interference with vesicle formation. Some HDL was found in the recon-

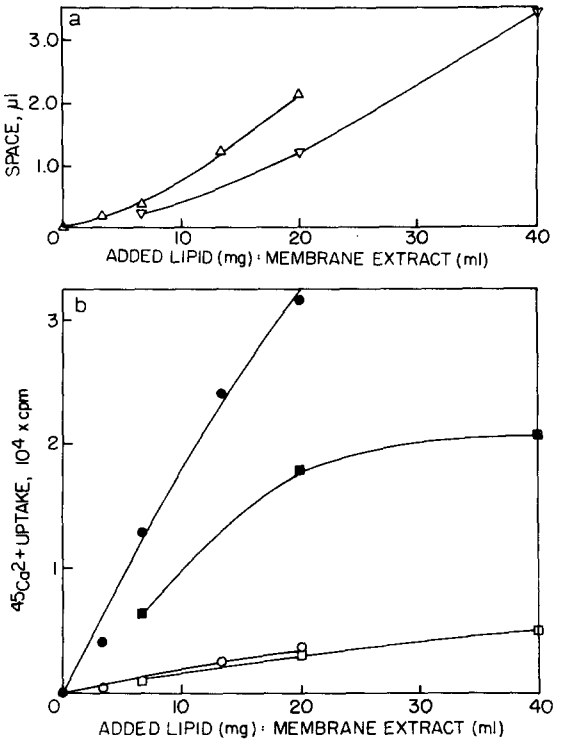

Fig. 5. The effect of the lipid/membrane extract ratio on reconstitution. Various amounts of lipid (in 0.25 ml) were added to 0.75 ml of extract. 10 µCi [^{3}H]choline/ml was present as 'trapped space' marker. $^{45}Ca^{2+}$ uptake was from 'trace' Ca^{2+} medium (1.5 µCi/ml) for 15 min at 39°C. The ratio, mg lipid/ml extract is plotted on the abscissa. (The ratio routinely used, see Materials and Methods, was 6.67.) Two experiments are plotted separately. (a) (upper) Shows the effect of the ratio on trapped space. (b) (lower) Shows ATP-dependent Ca^{2+} uptake. Filled symbols, +ATP; open symbols, —ATP.

stituted vesicle fraction. We do not know whether it is incorporated into the bilayer. However, much HDL together with a little membrane protein was eluted from the Bio-Gel column only a few ml after the vesicles, suggesting the formation of large HDL-lipid (and membrane protein) complexes. We could increase $^{45}Ca^{2+}$ transport activity/mg input membrane protein approx. 2.5-fold by increasing the lipid/membrane extract ratio (and therefore the lipid/HDL ratio), but at the cost of a modest decrease in the $^{45}Ca^{2+}$ +ATP/—ATP uptake ratio.

Acknowledgments

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