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Effect of centrifugation on separation by aqueous two-phase partition of an early and late endosome model using inside-out plasma membrane vesicles from plants

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Abstract

Inside-out vesicles of plasma membranes prepared from a plant source were used as models to investigate effects of centrifugal forces on separations of early and late endosome populations by aqueous two-phase partition. Endosome subpopulations were resolved readily by preparative free-flow electrophoresis where acidification of the interiors of late endosomes occurred upon addition of ATP to activate a proton translocating ATPase. The resultant increased diffusion potential provided for a surface difference between late and early endosomes to permit electrophoretic separation. With the plant membranes, unincubated inside-out plasma membrane vesicles modeled early endosomes, whereas inside-out vesicles incubated with 1 m*M* ATP modeled late endosomes. A latent, 2,4-dichlorophenoxyacetic acid (2,4-D)-(auxin)-stimulated NADH:protein disulfide reductase measured spectrophotometrically was used as an enzymatic marker for both populations of inside-out vesicles. Phase partition behavior of each population was quantitated using total protein as the parameter.

Keywords: Aqueous two-phase systems; Endosomes; Centrifugation

1. Introduction

The endosome/lysosome compartment of mammalian cells is highly heterogeneous [1–3]. Components are broadly classified as early, intermediate and late endosomes plus dense lysosomes. As detailed in a companion paper [4], understanding of the complex functional roles of endosomes in disease and drug response as well as normal cell functioning would be facilitated greatly by improved methods to resolve and fractionate the different endosome compartments.

We have previously separated early and late endosomes by preparative free-flow electrophoresis [5]. The purpose of the present studies was to determine to what extent these separations can be duplicated by aqueous two-phase partition and to determine if imposed centrifugal forces could influence the separation of different classes of endosomes. To facilitate these analyses, a model system was developed that would be expected to duplicate the electrophoretic behavior of early and late endosomes.

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Early endosomes are formed by invagination of the plasma membrane. They are, for practical purposes, inside-out plasma membrane vesicles. Therefore, inside-out plasma membrane vesicles were used to model early endosomes. Late endosomes have acquired the ability to acidify their interiors through ATP-driven proton afflux. Plant plasma membranes actively pump protons to the exterior of the cell and when converted into inside-out vesicles and incubated with ATP, model late endosomes. Therefore inside-out vesicles from plants provide a useful model for both early (−ATP) and late (+ATP) endosomes. In the model, inside-out plasma membrane vesicles, prepared from right side-out vesicles by freezing and thawing and shearing by passage through a 28.5 gauge needle fitted to a syringe, were used as a model to represent endosomes. Inside-out vesicles partitioned into the lower phase of a 6.4% PEG 3350–Dextran T-500 system, whereas right side-out vesicles remained in the upper phase.

2. Materials and methods

2.1. Isolation of plant membranes

Seeds of soybean (*Glycine max* L. Merr. var. Williams) were soaked in tap water 4 to 6 h, planted in moist vermiculite, and grown 4 to 5 days in darkness. Segments of 2 cm long, cut from the hypocotyl 5 mm below the cotyledons, were harvested under normal laboratory conditions and used for isolation of membranes. The hypocotyl segments (25 g) were homogenized in 100 ml of a medium containing 25 m*M* tris-2-(*N*-morpholino)ethanesulphonic acid (MES) (pH 7.5), 300 m*M* sucrose, 10 m*M* KCl and 1 m*M* $MgCl_2$ using a blender. After filtration through one layer of Miracloth (Chicopee Mills, NY, USA), the filtrate was centrifuged for 10 min at 6000 *g* (6000 rpm, Sorvall, HB-4 rotor), and the pellet was discarded. The resulting supernatant was centrifuged for 30 min at 40 000 *g* (Beckman, SW-28 rotor), and the supernatant was discarded. The 40 000 *g* pellets were resuspended in homogenization buffer as the source of material for aqueous two-phase partition [6].

2.2. Isolation of right side-out plasma membrane vesicles by aqueous two-phase partition

One gram of soybean microsomal membrane suspension was added to the two-phase system to give a final composition of 6.4% (w/w) Dextran T-500 (Pharmacia) and 6.4% (w/w) Carbowax (polyethyleneglycol) 3350 (Fisher Scientific), 0.25 *M* sucrose and 5 m*M* potassium phosphate, pH 6.8. The system was mixed by inversion 40 times in 30 ml glass centrifuge tubes and separated into two phases by centrifugation (1000 *g* for 5 min). The upper phase, enriched in plasma membranes, was partitioned twice against a fresh lower phase and separated by centrifugation as before. The lower phase was repartitioned against a fresh upper phase, followed by a fresh lower phase to collect and wash additional plasma membranes. Finally, all upper phases were combined, diluted with cold distilled water or with 5 m*M* potassium phosphate buffer, pH 6.8, and centrifuged in a SW-28 rotor at 95 000 *g* for 45 min to pellet the isolated plasma membranes, which were finally suspended in 0.5 to 1 ml of 25 m*M* Tris-MES containing 0.25 *M* sucrose or distilled water.

2.3. Isolation of inside-out plasma membrane vesicles produced by freezing and thawing

Plasma membrane vesicles, frozen and thawed four times to invert some of the vesicles [7], were sheared by 55 excursions through a 28.5 gauge needle fitted to a syringe and subfractionated by repeating the phase partition step. The lower phase was enriched in inside-out vesicles. The upper phase contained the remaining right side-out vesicles. The vesicles were collected by centrifugation at 95 000 *g* for 45 min and resuspended in 25 m*M* Tris-MES, pH 7, containing 0.25 *M* sucrose.

2.4. Incubations with ATP

To induce negative diffusion potentials, preparations were incubated for 15 min at room temperature in the absence or presence of 1 m*M* ATP contained in 2.5 m*M* Tris-MES (pH 7.0), 50 m*M* chloride (KCl or NaCl), 2 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol

(DTT), and 75 to 100 μg protein in a total volume of 1.5 ml as indicated.

2.5. Preparative free-flow electrophoresis

The conditions for preparative free-flow electrophoresis were: flow-rate 2 ml/fraction/h, injection rate 2.5 ml/h, the voltage 1150 V and amperage ca. 175 mA. Just before injection, 1.5 ml of chamber buffer (10 m*M* acetic acid, 10 m*M* triethanolamine, 0.25 *M* sucrose, 0.5 m*M* $MgCl_2$ and 5 m*M* glucose, pH 6.5, was added to the mixture. After electrophoresis, the absorbance of 2 ml fractions was determined at 280 nm. Based on absorbance, fractions were combined and the endosomes collected by centrifugation (20 000 *g*, 30 min).

2.6. Centrifugation during resolution of phases

To test the response of the two phase partitioning to imposed centrifugal forces, the two-phase samples were centrifuged in a refrigerated (4°C) swing-out rotor (Sorvall HB-4) or, after transfer to 13×100 mm disposable borsilicate glass tubes, at room temperature (IEC HN-SII table top centrifuge) for 5 min during phase resolution. Uncentrifuged, paired samples were covered with parafilm and rested horizontally at the same temperature as during centrifugation (on ice for 4°C, bench top for room temperature) in place of the centrifugation.

2.7. Determination of protein

Protein content was determined by the bicinchominic acid/copper assay (BCA) [8] obtained from Pierce. Standards were prepared with bovine serum albumin.

2.8. Measurement of NADH oxidation by plasma membrane vesicles

NADH oxidation was measured spectrophotometrically using a Hitachi U3210 spectrophotometer from the decrease in absorbance at 340 nm. The assays were at 24°C with stirring by a magnetic stirring assembly. Individual reaction mixtures contained 1 m*M* KCN, 50 m*M* Tris-MES, pH 7, and 40 to 50 μg plasma membrane protein. After a 3 min equilibration period, 150 μ*M* NADH was added. Reaction rates at a full scale absorbance of 0.06 were recorded with a linear recorder. The rates of NADH oxidation were calculated using a millimolar absorptivity of 6.22 for NADH. Samples stored on ice in a cold cabinet or frozen for more than 48 h required the addition of 1 μ*M* GSH for 10 min followed by 0.03% hydrogen peroxide or 100 μ*M* GSSG to restore the NADH oxidase activity.

3. Results

3.1. Characteristics of inside-out plasma membrane vesicles

The inside-out vesicles, prepared from right side-out plasma membrane vesicles from hypocotyls of dark-grown seedlings of soybean, were >90% plasma membrane derived based on analyses of marker enzymes and specific cytochemical stains [9]. The plasma membrane content of the preparations was verified by reaction with phosphotungstic acid at low pH as described [10]. Marker enzymes were as described [9]. Vesicle orientation was confirmed from measurements of ATP latency and from electrophoretic mobility [11].

Vesicles were everted by several cycles of freezing and thawing and passage after each freeze–thaw cycle through a 28.5 gauge needle. To separate right side-out and inside-out vesicles a 6.4% PEG 3350–Dextran system was used. The inside-out vesicles partitioned into the lower Dextran-rich phase whereas the right side-out vesicles remained in the PEG 3350-rich upper phase.

3.2. Preparative free-flow electrophoretic separation of inside-out plasma membrane vesicles incubated with and without ATP

When plant homogenates were incubated with ATP, the electrophoretic mobility of tonoplast (vacuole membranes) was enhanced [4]. These vesicles, which were oriented cytoplasmic side out, were active in acidification of their interiors and this acidification was ATP-dependent [9]. It should be

possible to observe a similar shift with inside-out plasma membrane vesicles since these vesicles also are capable of ATP-dependent acidification of their interiors [5].

Purified fractions of inside-out plasma membrane vesicles were analyzed by preparative free-flow electrophoresis, both before (−ATP) and after (+ATP). The ATP-incubated vesicles, which carried a negative diffusion potential, exhibited a significantly greater electrophoretic mobility (five electrophoretic fractions) than the unincubated vesicles (Fig. 1).

A result similar to that with the inside-out plasma membrane vesicles was observed with rat [5] and bovine [4] kidney endosomes and endosomes from HeLa cells [4]. The early endosomes which did not acidify their interiors when incubated with ATP exhibited low electrophoretic mobility. When incubated with ATP, a second population representing late endosomes with acidified interiors and a negative diffusion potential outside appeared. Thus, the unincubated inside-out plasma membrane vesicle model early endosomes and the ATP-incubated, inside-out plasma membrane vesicles model late endosomes.

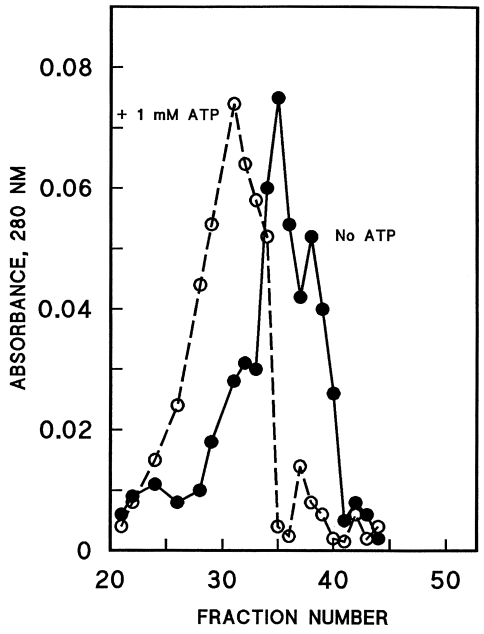

Fig. 1. Free-flow electrophoretic separation of inside-out plasma membrane vesicles prepared by aqueous two-phase partition. In the absence of ATP, the distribution of vesicles was symmetrical and of low electrophoretic mobility (−ATP). When the preparations were incubated for 15 min with 1 m*M* ATP, ATP-induced enhanced electrophoretic migration of the particles modeling that of late endosomes with acidified interiors was observed (+ATP). Fractions are numbered from the anode. Results are numerical averages of 3 experiments.

3.3. Aqueous two-phase partition of inside-out plasma membrane vesicles before and after incubation with 1 mM ATP

To select a two-phase polymer concentration for the centrifugation experiments, the inside-out vesicles were incubated for 15 min either in the presence or absence of 1 m*M* ATP as described in Section 2 and then subjected to a second aqueous two-phase partition step (Fig. 2). When separated as a function of different polymer concentrations, optimum partition into the lower phase was obtained with a 6.4% phase system (Fig. 2).

As the polymer concentration was reduced, some of the vesicles entered the upper phase. When the polymer concentrations were 5.8% or less, most of the inside-out vesicles tended to partition into the upper phase. Although a slight tendency to be retained in the lower phase was noted on average for endosomes where the interiors were acidified by incubation with ATP, the partition coefficients were not significantly affected by incubation with ATP at any of the polymer concentrations examined (Fig. 2).

3.4. Response to centrifugation of aqueous two-phase partition of inside-out plasma membrane vesicles before and after incubation with 1 mM ATP

Since centrifugation during phase separation enhanced the transfer of HeLa endosomes into the upper phase at a 5.8% polymer concentration [4], the effect of centrifugation was studied on partition of the inside-out soybean vesicles with 5.2 or 5.4% (w/w) polymer-containing phase systems and with 5.8 and 6.0% phase systems. The 5.2 or 5.4% phase systems resolved much more slowly than the 5.8 and 6.0% phase systems and the effect of a brief, low speed centrifugation was to decrease the amount of vesicles in the upper phase in the absence of ATP whereas in the presence of ATP, centrifugation was

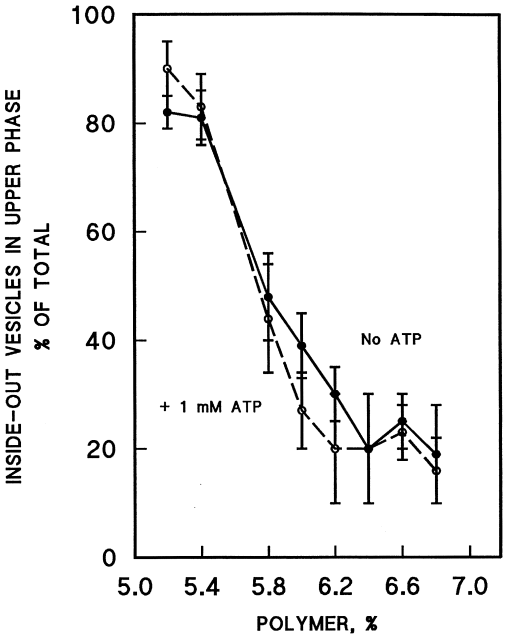

Fig. 2. Partitioning of inside-out plasma membrane vesicles from hypocotyls of dark-grown soybean seedlings as a function of phase composition using a PEG-3350–Dextran system. The vesicles are initially separated from right side-out vesicles by partitioning into the lower phase of a 6.4% system. As the polymer concentration was decreased or increased from 6.4% some of the inside-out vesicles entered the upper phase (solid symbols). If the interiors of the inside-out vesicles were first acidified by incubation with 1 m*M* ATP (+ATP) to mimic late endosomes, the tendency to remain in the lower phase with decreasing but not increasing polymer concentration was slightly enhanced (open symbols, dotted line). Results are averages of 3 to 5 determinations at each polymer concentration±standard deviations.

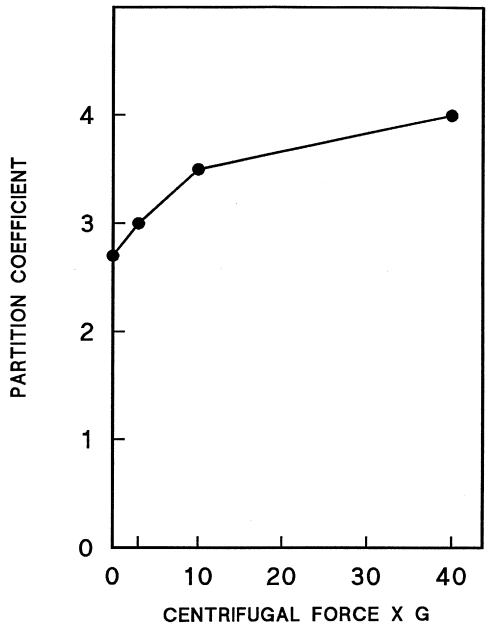

Fig. 3. Effect of centrifugation on partition coefficient of inside-out plasma membrane vesicles in a 5.4% phase system. The tendency for the vesicles to favor the upper phase was enhanced toward a plateau at about 40 g.

without effect. Therefore, centrifugation experiments were continued only with the 5.8 and 6.0% systems. Although centrifugation caused more of the endosomes to partition into the upper phase (Fig. 3), the response was less than with HeLa endosomes. Centrifugation did not appear to enhance separation of the acidified and unacidified vesicles by phase partition (not illustrated).

3.5. Latent auxin-stimulated oxidation of NADH as a marker for inside-out plasma membrane vesicles

Plasma membrane vesicles from plants exhibit an NADH oxidase activity at both surfaces of the plasma membrane [12]. However, the two activities exhibit different properties. One difference with utility in the present investigation is that the activity of the right side-out vesicles was stimulated by plant growth factors termed auxins [12]. Both natural (indole-3-acetic acid) and synthetic (2,4-dichlorophenoxyacetic acid=2,4-D) auxins affected the activity [13]. The NADH oxidase activity of right side-out vesicles was stimulated about 70% by 2,4-D, whereas that of the inside-out vesicles was not stimulated (Table 1). With inside-out vesicles, the 2,4-D stimulated activity was on the inside of the vesicle and latent. As NADH is an impermeant substrate, the inside-out vesicles must be opened in order to demonstrate the activity.

Inside-out plasma membrane vesicles when ruptured by Triton X-100 showed enhanced 2,4-D stimulated activity whereas right side-out vesicles did not (Table 1). In the presence of 0.1% Triton X-100, both the right side-out and inside-out vesicles exhibited an auxin-responsive oxidation of NADH which was approximately equal to the calculated sum

Table 1
2,4-D response of right side-out and inside-out plasma membrane vesicles prepared by freezing and thawing followed by aqueous two phase separation and response to 0.01% Triton X-100 (from DeHahn et al. [12])

Vesicle orientation	NADH oxidase (nmol/min mg protein)	
	No addition	+1 μ*M* 2,4-D
(A) No Triton X-100		
Right side-out	2.9±0.5	4.9±0.6
Inside-out	9.3±1.2	9.8±1.5
(B) +0.01% Triton X-100		
Right side-out	11.8±0.7	15.2±1.9
Inside-out	12.0±1.0	15.7±0.9
(Calculated from A; right side-out+inside-out)	12.2	14.7

Results are averages from four experiments±standard deviations.

of the two activities (inside-out plus right side-out) in the absence of Triton X-100 (Table 1).

4. Discussion

The inside-out plasma membrane vesicles of hypocotyls of dark grown soybean seedlings appear to model very well the electrophoretic properties of early endosomes and, when incubated with 1 m*M* ATP, those of late endosomes. Therefore, the inside plasma membrane vesicles were utilized in studies to attempt to effect the separation of early and late endosomes by aqueous two-phase partition.

Initially, the inside-out vesicles partitioned into the lower phase of a 6.4% PEG 3350–Dextran T-500 system whereas right side-out vesicles remained in the upper phase. The phase partition behavior of the vesicles incubated in the absence or presence of 1 m*M* ATP over the wide range of polymer concentrations showed no significant effect of the incubation with ATP.

These data indicate that it may be much more difficult to separate early and late endosomes or endosome models by two-phase partition compared to preparative free-flow electrophoresis. The reasons for this have been addressed previously [5]. Our findings show that a common parameter among free-flow electrophoretic separations may be the ability to create an (outside) negative diffusion potential in addition to charged molecules at the cell surface. This is necessary to explain why membrane composition and electrophoretic mobility are not closely correlated and why different vesicles of very similar composition (e.g., vesicle membranes incubated in the absence or presence of ATP) can be separated by preparative free-flow electrophoresis.

Unfortunately, a diffusion potential of itself does not alter electrophoretic mobility of a particle in a buffered solution. A mobile ion cloud around the particle, the so-called Debye–Hückel layer [14,15] is generally considered to determine electrophoretic mobility. The Debye–Hückel layer is defined as the outer region of an electric double layer of ions surrounding the particles. In an electric field, this layer becomes deformed. The outer ion cloud layer is shifted in a direction opposite to that of particle movement. As a result, underlying charged groups such as those generated by a negative diffusion potential might contribute to electrophoretic mobility but not to separation by aqueous two-phase partition.

Thus we have argued that organelle separations not possible by aqueous two-phase partition are possible by free-flow electrophoresis because of the specific deformation of the Stern layer relating to ζ potential. The ζ potential, the actual change of a particle, is determined at the Stern layer which is the term given to the surface of shear between the inner and outer portion of the double layer of absorbed ions. Because separations of early and late endosomes may depend on diffusion potential generated by ATP-dependent acidification of vesicle interiors, techniques are under development whereby aqueous two-phase separations will be carried out in an

electric field [16]. The utility of electrophoresis in combination with aqueous two phase polymer solutions has been demonstrated previously for separation of red blood cells from Alzheimer patients [17,18].

As an enzymatic marker for the inside-out plasma membrane vesicles, we have developed an assay based initially on the observation that the external plasma membrane surface exhibited a 2,4-D-stimulated oxidation of NADH whereas the internal plasma membrane surface did not [12]. This meant that with inside-out vesicles the 2,4-D stimulated activity would be latent and observed only after the vesicles were disrupted, for example, with low concentrations of a detergent such as 0.1% Triton X-100. The latent 2,4-D-stimulated oxidation of NADH thus provides a convenient enzymatic marker to evaluate the distribution of inside-out plasma membrane vesicles after aqueous two-phase partition. To assay, two reactions are carried out. One is in the presence of 1 μM 2,4-D but in the absence of 0.1% Triton X-100. The other is in the presence of both 1 μM 2,4-D and 0.1% Triton X-100. 2,4-D-stimulated activity resulting from Triton X-100 addition is then taken as a measure of the population of inside-out vesicles present.

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