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Purification of rabbit lacrimal gland plasma membranes by aqueous two-phase affinity partitioning

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Abstract

We describe the purification of lacrimal gland plasma membranes by affinity partitioning using a two-phase system containing polyethylene glycol and dextran in which wheat germ agglutinin conjugated to dextran is used as affinity ligand. When partitioning a microsomal fraction, the plasma membrane marker 5′-nucleotidase was obtained in the affinity ligand-containing bottom phase, whereas the endoplasmic reticulum marker NADH-ferricyanide reductase remained in the top phase. The affinity partitioning behaviour of components involved in exocytosis and cellular signalling was also examined.

Keywords: Aqueous two-phase systems; Affinity partitioning; Lacrimal gland; Phosphatidylinositol 4-kinase

1. Introduction

Purification of plasma membranes is often performed using combinations of differential and density gradient centrifugations. Fractions thus obtained can be further purified and analysed by counter-current distribution in aqueous two-phase systems [1,2]. However, both density gradient fractionation and counter-current distribution are rather time-consuming and thereby less suitable for the study of rapid biological processes such as protein translocation from intracellular sites to plasma membranes or phosphorylation/dephosphorylation events in connection with cell stimulation.

Affinity partitioning might be an attractive alternative to these traditional methods. Ideally, the affinity ligand coupled to one of the phase polymers will selectively pull the membrane fraction of interest from one phase of a two-phase system to the ligand-containing phase allowing a one-step purification making the method much more rapid than conventional ones. This technique has been used previously to purify acetylcholine receptors from *Torpedo californica* [3] and opiate receptors from bovine brain cortex [4]. Also, wheat germ agglutinin (WGA) coupled to dextran was used to purify plasma membranes from rat liver and hepatocytes [5,6] taking advantage of the heavy glycosylation of the plasma membrane allowing the lectin to bind to *N*-acetylglucosamine and sialic acid exposed on the membrane surface.

In the present study we have explored the possibility to use WGA as a two-phase affinity ligand for the partitioning of plasma membranes from primary cultures of rabbit lacrimal gland acinar cells as well as from lacrimal gland tissue. Earlier, subcellular fractionations of rat and rabbit lacrimal glands have

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been performed using a combination of isopycnic centrifugation and two-phase counter-current distribution [7,8]. This approach was also used to study the carbachol-stimulated translocation of several components, including Na^+,K^+-ATPase, from intracellular stores to plasma membranes in lacrimal glands [9–12]. It will be shown that these plasma membranes can be isolated by two-phase affinity partitioning using WGA as the affinity ligand, suggesting a more facile way of studying rapid processes involving plasma membranes.

2. Experimental

2.1. Materials

Female NZW rabbits weighing approximately 2 kg were obtained from ESF-Products (Estuna AB, Norrtälje, Sweden), and handled in compliance with the Guide to the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and the Swedish National Board for Laboratory Animals. Bovine serum albumin (fraction V), soybean trypsin inhibitor, the insulin–transferrin–sodium selenite media supplement, hydrocortisone, Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) and wortmannin were from Sigma (St. Louis, MO). Ham's nutrient mixture F-12, DME (low-glucose Dulbecco's modified Eagle's medium), Pen-Strep-Glu and collagenase were purchased from Gibco-BRL (Grand Island, NY). DNase I (bovine pancreas) was obtained from Calbiochem (La Jolla, CA), hyaluronidase from Worthington (Freehold, NJ), and Matrigel was from Collaborative Biomedical Products (Becton-Dickson, Bedford, MA). Dextran T500 and [^{3}H]AMP were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Polyethylene glycol (PEG) 3350 was from Union Carbide (Danbury, CT), wheat-germ agglutinin (WGA) from Roche Molecular Biochemicals (Basel, Switzerland) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) from Synthelec AB (Lund, Sweden). Silica gel 60 plastic TLC plates (20×20 cm) (Merck, Darmstadt, Germany) were pretreated with 1% potassium oxalate in 50% (v/v) ethanol for 1 min, left to dry in a fume hood overnight and cut in three 6.7-cm pieces before use. All other chemicals were reagent grade and were obtained from standard suppliers.

2.2. Preparation of cells and subcellular fractionation

Single acinar cells from the intraorbital lacrimal glands were isolated as described earlier [12,13]. The cells obtained were washed and cultured for 40 h in six-well plates at a cell density of 8×10^6 cells/well using serum-free medium supplemented with 40 μg/ml Matrigel allowing the cells to re-organise into acinar-like structures.

2.2.1. Microsomal membranes from cells

The culture medium was carefully aspirated off the wells and each well was rinsed with ice-cold homogenisation medium containing 0.25 *M* sucrose, 1 m*M* phenylmethylsulfonyl fluoride, 0.5 m*M* Na-EDTA and 5 m*M* Tris–HCl, pH 8.0. The cells were harvested with a rubber policeman and the cell suspension was centrifuged at 100 *g* for 5 min. The resulting pellet was resuspended in homogenisation medium and homogenised 10 times in a tight-fitting hand-driven Potter-Elvehjem homogeniser. The homogenate was centrifuged at 2200 *g* for 10 min at 4°C. The supernatant was collected and the pellet rehomogenised and centrifuged as above. The supernatants were combined and centrifuged at 75 000 *g* for 2 h at 4°C. The resulting microsomal pellet was resuspended in homogenisation medium and frozen at −80°C.

2.2.2. Microsomal membranes from tissue

The intraorbital lacrimal glands from one rabbit were excised and cut into 1-mm^3 pieces with a pair of scissors. The fragments were transferred to a glass–glass homogeniser and homogenised 10 times with a loose-fitting pestle in 5 ml homogenisation medium. The homogenate was filtered through four layers of gauze and centrifuged at 2200 *g* for 10 min at 4°C. The resulting supernatant was collected, the pellet re-extracted as above using a tight-fitting pestle and the two supernatants were combined. The subsequent steps were identical to those for isolated cells.

2.3. Affinity two-phase partitioning

Wheat germ agglutinin (WGA) was coupled to dextran T500 after activation with tresyl chloride [6] using dried solvents [14]. The degree of coupling was 2.3 mg WGA/g dextran as determined according to Bradford [15] using free WGA as standard.

Two-phase systems were prepared in 3-ml plastic Ellerman tubes and equilibrated at 4°C [14]. The systems had a final weight of 2 g including the sample, and contained 6.0% (w/w) PEG 3350, 6.0% (w/w) Dextran T500 and 15 m*M* boric acid adjusted to pH 7.8 with Tris base. When WGA–dextran was added the amount of free dextran was decreased to keep the total dextran concentration constant.

After the addition of microsomal membranes the systems were mixed thoroughly by 20 inversions, 10 s vortexing and another 20 inversions followed by phase separation. This was facilitated by centrifugation at 150 *g* for 5 min in a table-top centrifuge. The top phase was siphoned off leaving the interface with the bottom phase. The phase volumes were determined by marking the phase boundaries and weighing in the volumes with water.

2.4. Assays

The following enzyme assays were performed: 5′-nucleotidase, a plasma membrane marker [16], NADH-ferricyanide reductase, an endoplasmic marker [17] and Na^+,K^+-ATPase [18]. Phosphatidylinositol (PI) 4-kinase activity was assayed essentially as described [19]. The incubation mixture contained in a final volume of 50 μl: 50 m*M* Hepes–KOH, pH 7.5, 10 m*M* $MgCl_2$, 0.5 m*M* PI, 0.25% (w/v) Triton X-100, 1 m*M* [γ-^{32}P]ATP (approx. 1.5 Bq/pmol) and membrane sample (up to 20 μg protein). Incubations were for 1–2 min at 25°C. After termination and methanol–HCl washes [19] the reaction products were separated on oxalate-treated TLC plates (see Section 2.1) using chloroform–methanol–water–25% ammonia (45:45:10.5:3.5, v/v/v/v) as the mobile phase. Radioactive spots were visualised and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

To measure wortmannin-insensitive and -sensitive PI 4-kinase activities all samples were assayed both with and without 10 μ*M* wortmannin. The activities were calculated assuming that insensitive PI 4-kinases are unaffected and sensitive ones inhibited to 90% by the added wortmannin (unpublished results and [20]). Protein was determined according to Bradford [15] using bovine serum albumin as standard. Top and bottom phases with appropriate amounts of WGA were used as blanks for protein determinations of the two-phase systems.

3. Results and discussion

Conditions were chosen to partition the bulk of microsomal membranes from lacrimal glands or isolated lacrimal cells in the PEG-rich top phase of a PEG–dextran two-phase system. When increasing concentrations of WGA–dextran was added to such a system also containing microsomes from isolated lacrimal cells the plasma membrane marker 5′-nucleotidase was gradually redistributed from the PEG-rich top phase to the WGA–dextran containing bottom phase (Fig. 1). Thus, without added affinity ligand approximately 90% of the enzyme marker partitioned in the top phase, while at 50 μg WGA bound to dextran, and above, 85–90% instead partitioned in the bottom phase. In contrast, the endoplasmic reticulum marker NADH-ferricyanide reductase remained in the top phase at all concentrations of WGA tested, whereas the total protein redistri-

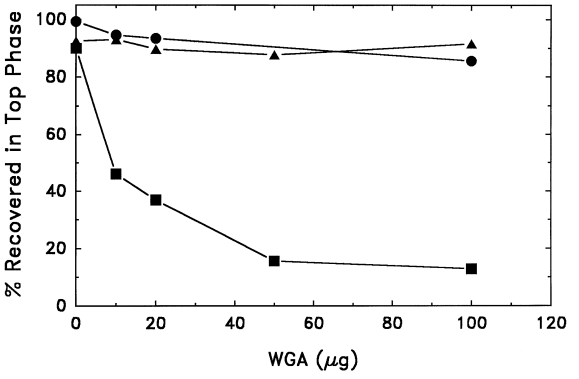

Fig. 1. Effect of increasing amounts of WGA–dextran on partitioning of microsomal membranes from lacrimal cells. Membrane suspension was added to 2-g systems (see Section 2) containing the indicated amounts of WGA coupled to dextran. Protein (●), NADH-ferricyanide reductase (▲) and 5′-nucleotidase (■).

buted to a slight extent into the bottom phase in the presence of WGA presumably reflecting the redistribution of plasma membranes. Thus, plasma membranes were selectively pulled to the bottom phase upon introduction of the affinity ligand, while intracellular membranes were retained in the top phase, as illustrated here by the endoplasmic reticulum marker. The enrichment of the plasma membrane marker after the affinity partitioning step was approximately 10-fold compared to the microsomal preparation.

Microsomal membranes prepared from lacrimal gland tissue were also partitioned both without affinity ligand and with 100 μg WGA coupled to dextran in the two-phase system. The distribution of protein and 5′-nucleotidase activity was the same as in the corresponding measurements of Fig. 1 (results not shown), indicating that the affinity method works equally well with membranes obtained from whole tissue and isolated cells. These results are consistent with earlier affinity partitioning experiments performed on microsomal fractions from rat liver and hepatocytes, where plasma membrane markers in both cases redistributed to the same extent as here from top to bottom phase upon introduction of WGA–dextran leaving markers for the endoplasmic reticulum as well as Golgi membranes and endosomes mainly in the top phase [5].

To explore the potential usefulness of the affinity method we also examined the partitioning of Na^+,K^+-ATPase and of PI 4-kinases with and without the affinity ligand. The subcellular distribution of Na^+,K^+-ATPase in lacrimal cells is of interest to follow as this enzyme partly redistributes from internal membranes to plasma membranes upon cell stimulation [12]. PI 4-kinases, of which there are wortmannin-insensitive and -sensitive forms, are also of interest as they might redistribute during exocytosis [21] or be activated after hormone stimulation [22]. They have also been implicated in membrane budding and fusion events [23].

The Na^+,K^+-ATPase activity showed an intermediate distribution in the affinity system compared to that of markers for plasma membrane and endoplasmic reticulum (Fig. 2), approx. 60% being pulled to the bottom phase in the presence of the affinity ligand. This implies a partial plasma membrane localisation of the enzyme in the unstimulated cell, consistent with earlier results [12].

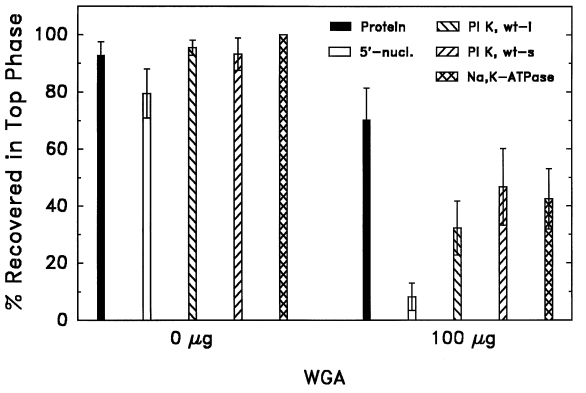

Fig. 2. Affinity partitioning of microsomal membranes from lacrimal cells. Membrane suspension was partitioned in 2-g systems (see Section 2) either without or with 100 μg WGA coupled to dextran. Protein, 5′-nucleotidase, wortmannin-insensitive (wt-i) PI kinase, wortmannin-sensitive (wt-s) PI kinase and Na^+,K^+-ATPase as indicated. The data are based on six preparations (±SD), except Na^+,K^+-ATPase which is based on two preparations.

Both the wortmannin-sensitive and -insensitive PI 4-kinase activities distributed similarly to the Na^+,K^+-ATPase (Fig. 2), suggesting that these enzymes also are partly localised in plasma membranes in the lacrimal cells examined. The wortmannin-insensitive enzyme in other tissues [21,24] is localised mainly in exocytotic membranes rather than in plasma membranes, even though it has been clearly shown that the wortmannin-insensitive type II PI 4-kinase associates with plasma membrane protein complexes and is probably involved in the restoration of the hormone-sensitive pool of PIP_2 [25]. The higher relative amount of wortmannin-insensitive activity in the lacrimal plasma membranes might reflect a different relationship between exocytosis and hormone stimulation in lacrimal cells compared to, for instance, liver cells [21]. However, this merits further investigations, as the precise function of PI 4-kinases in different locations is not known. A plasma membrane location of wortmannin-sensitive PI 4-kinase is rather interesting. This type of activity has been shown to participate in a late phase in the restoration of the hormone-sensitive pool of PIP_2 in adrenal glomerulosa cells [22], but so far no wortmannin-sensitive PI 4-kinase activity has been identified in plasma membranes.

The present results indicate that affinity two-phase partitioning using WGA as the affinity ligand can be

used for the isolation of lacrimal plasma membranes. The method would allow the facile study of the localisation of components to the plasma membrane. In these preliminary studies, we have explored the possibility to use the affinity method for localisation studies of components involved in cellular transport and signalling events. It will now be of interest to study time-resolved events involving lacrimal cell plasma membranes following various kinds of stimulation.

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