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Flow Cytometry and Sorting of Amphibian Bladder Endocytic Vesicles Containing ADH-Sensitive Water Channels

F.G. van der Goot†, A. Seigneur‡, J.-C. Gaucher‡, and P. Ripoche§
†European Molecular Biology Laboratory, D-6900 Heidelberg, Germany, ‡DEIN/SIR,§ Département de Biologie Cellulaire et Moléculaire, C.E.N. Saclay, 91191 Gif-sur-Yvette, France

Summary. The water permeability of ADH target epithelial cells is believed to be regulated by a cycle of exo-endocytosis of vesicles containing functional water channels. These vesicles were selectively labeled in intact frog urinary bladders with an impermeant fluorescent marker, 6-carboxyfluorescein. Vesicle suspensions containing the labeled endosomes were obtained by homogenization and differential centrifugation of bladder epithelial cells. The osmotic permeability of the endocytic vesicles was measured, using a stopped-flow fluorescence technique, in the absence or in the presence of $HgCl_2$. This permeability was found very high (500 μm/sec) and inhibited by 1 mM $HgCl_2$ (90%), thus confirming the presence of water channels. The labeled endosomes were then separated from the other membrane vesicles by flow cytometry and sorting. Their protein content was analyzed by electrophoresis on ultrathin polyacrylamide gels. Two double bands were found at 71 and 55 kDa as well as a small band at 43 kDa. They respectively correspond to 31, 38 and 10% of the total amount of silver-stained proteins present in the sorted endosomes, while they only represent 2, 4, and less than 1% of the proteins contained in the vesicle suspension, before sorting. These highly enriched proteins (or at least one of them) are likely to be involved in the mechanism of water transport. Associated to their partial purification by differential centrifugation, the sorting of the endosomes by flow cytometry seems a good way to further characterize the water channel.

Key Words water channels · amphibian urinary bladder flow cytometry · endocytosis · ADH

Introduction

Over the past decade, the overall mechanism regulating the transepithelial water permeability of amphibian urinary bladder has become quite clear: in the absence of antidiuretic hormone (ADH) the epithelium is remarkably impermeable to water (Hays & Leaf, 1962); the appearance of serosal vasopressin triggers an increase of the apical membrane water permeability (Hays & Leaf, 1962; Hays & Franki, 1970), the permeability of the basolateral membrane remaining permanently very high (van der Goot, Corman & Ripoche, 1991). Various studies converge to the idea that the increase in luminal water permeability results from the fusion of intracellular vesicles, containing water channels, with the apical membrane of granular cells (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Casey & DiScala 1978; Muller, Kachadorian & DiScala, 1980; Wade, Stetson & Lewis, 1981; Brown, Grosso & DeSousa, 1983; Harris, Wade & Handler, 1986; Shi & Verkman, 1989; for review *see* Handler, 1988). This process seems reversible: after ADH removal, an endocytosis of patches of apical membrane was observed concomitant with a decrease in water permeability (Coleman, Harris & Wade, 1987; Harris, Handler & Blumenthal, 1990). Recently, this hypothesis was confirmed by Shi and Verkman (1989) and later by Harris et al. (1990) who showed that the ADH-induced endocytic vesicles contain densely packed water channels. Indeed these authors have measured the kinetics of water efflux on fluorescent labeled endosomes using a stopped-flow fluorescence technique. They have observed that the water permeability of these vesicles was extremely high, sensitive to mercurial chloride and that the activation of energy of water movement was low (4 kcal/mol), three characteristics supporting the presence of water channels (Macey, 1984).

As these endosomes have a very high water channel density and can be selectively labeled, they seem a good starting point for the search of the protein, or proteins, that are involved in the water transport mechanisms. The two main strategies to isolate them from the rest of the urinary bladder epithelial cell are: (i) differential centrifugations of crude homogenates (Kessler et al., 1978; Courtoy, Quintart & Bauduin, 1984) and identification of the fluorescent fraction; (ii) sorting the fluorescent vesicles from the rest of the suspension. The first possi-

bility, which is very difficult to adapt as the ADH-induced endocytic vesicles represent only a small fraction of the total cellular membranes, was attempted by Harris and co-workers (1987). Their endosome-enriched fraction still contained a great number of different proteins, which could not all be involved in the water transport mechanism. In the present study, we chose to sort the fluorescent labeled endosomes by flow cytometry. With this technique, the vesicle suspension was analyzed vesicle-by-vesicle, enabling the identification and then the separation of the fluorescent endosomes (Wilson & Murphy, 1989).

Our overall procedure was: (i) in intact frog bladders, to label the endocytic vesicles, or endosomes, with an impermeant fluorescent marker, 6-carboxyfluorescein (6-CF), by inducing a specific endocytosis during the washout of ADH; (ii) to check by a stopped-flow fluorescence technique on a vesicle suspension of 6-CF-treated frog bladders that the labeled endosomes indeed had an uncommonly high water permeability inhibitable by mercurial salts; (iii) to identify the labeled endosomes by flow cytometry and then sort them from the rest of the vesicle suspension; (iv) to analyze in SDS-PAGE the protein content of the sorted endosomes.

This work was presented in abstract form at the 10th International Congress of Biophysics in Vancouver, Canada, in July 1990.

Materials and Methods

Labeling of ADH-Induced Endocytic Vesicles

Frog (*Rana esculenta*) urinary bladders were filled to capacity with a Ringer solution (in mM: NaCl, 112, KCl 5, $MgCl_2$ 1, $NaHCO_3$ 2.5, glucose 1, $CaCl_2$ 1), mounted as sacs on a polyethylene tubing (Biotrol n° 9, Paris, France) and suspended in a Ringer bath containing 20 mU/ml of oxytocin (Syntocinon, Sandoz, Switzerland). After 20 min of incubation at room temperature, the bladders were emptied and filled with a dilute Ringer (in mM: NaCl 5.6, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, tricine 5, titrated to pH 7.8 with NaOH) containing 2 mM 6-carboxyfluorescein (Sigma, St. Louis, MO). Bladders were incubated for 5 min with these solutions followed by an additional 10 min with zero oxytocin in the serosal bath, always at room temperature. All subsequent steps were carried out at 4°C. Both the apical and basolateral faces of each bladder were then rinsed extensively with Ringer solution. The epithelial cells were removed from the subepithelial tissues by 10 strokes of a Dounce homogenizer (pestle A) in the presence of 2 ml of an ice-cooled buffer containing 100 mM KCl, 5 mM tricine, 1 mM phenyl methyl sulfonide fluoride, 120 μM benzamidine, 1.46 μM pepstatin, 1.65 μM antipain, 10.5 μM leupeptin titrated to pH 7.4 with KOH. The subepithelial tissue was then removed. By 200 strokes of a Dounce homogenizer (pestle B), the plasma membrane of most epithelial cells was disrupted and the labeled endocytic vesicles were released. The membrane suspension thus obtained was centrifuged for 10 min at 100 × *g* to remove large debris, nuclei and unbroken cells. The extravesicular 6-CF was removed by passing the supernatant on a gel filtration column (PD-10, Pharmacia, Uppsala, Sweden). The vesicles are thus eluted in the void volume, whereas the probe is retained on the column. No sieving of the vesicles occurs during this procedure. The protein concentration of the filtered membrane suspension, determined according to the method described by Bradford (1976) with bovine serum albumin as standard, was between 1 and 3 mg/ml.

In view of the measurement of the size of the endosomes, ADH-induced endocytosis was measured using native ferritin (Sigma, St. Louis, 80 mg/ml) as a fluid phase marker instead of 6-CF. The bladder were then washed, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and prepared for electron microscopy observations as described by Rambourg et al. (1991).

Preparation of Unlabeled ADH-Induced Endocytic Vesicles

The above described protocol was repeated with the following modifications: (i) after a 20 min incubation in the presence of serosal ADH, the mucosal medium was replaced with a hypotonic Ringer (5.6 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM tricine, titrated to pH 7.8 with NaOH) that did not contain 6-CF; (ii) just before disrupting the bladder, 2 mM of 6-CF was added to the mucosal medium, the bladder was then immediately excised and rinsed in ice-cooled Ringer. The purpose of this final addition of 6-CF was to obtain the same extravesicular 6-CF concentration in the labeled and the unlabeled endosome preparations.

Osmotic Permeability Measurements

Stopped-flow fluorescence measurements were performed on Bio-Logic SFM2 (Echirolles, France). A 150 W halogen lamp was used for maximal stability of the light source. The sample was excited at 460 nm. Fluorescence was detected through a LP 520 filter (MTO J 526a, Massy, France).

Before performing stopped-flow experiments on 6-CF labeled endosomes, the feasibility of water efflux measurements was checked on kidney brush-border membrane vesicles. They were prepared as previously described (van der Goot, Podevin & Corman 1989) in a buffer containing 20 mOsm mannitol, 50 mM HEPES, pH 7.4 adjusted with KOH. To obtain 6-CF labeled brush-border membrane vesicles, the same protocol was applied except that 2 mM of the fluorescent marker was added to the preparation buffer. The labeled and the unlabeled preparations were mixed in order to have 4% of labeled vesicles. 0.1 ml of the suspension (0.4 mg protein/ml) was mixed with an equal amount of a hyperosmotic solution in order to reach a final inwardly directed 100 mOsm mannitol gradient. The induced fluorescence quenching was monitored as a function of time and recorded on an AT Tandon personal computer for subsequent analysis. The maximal acquisition rate was 0.1 msec/point.

The same experiment was performed on frog bladder vesicle suspension containing the labeled endosomes: the vesicles were submitted to an inwardly directed 150 mOsm gradient. This gradient was obtained by addition of sucrose to the KCl buffer (100 mM KCl, 5 mM tricine, 1 mM phenyl methyl sulfonide fluo-

ride, 120 μM benzamidine, 1.46 μM pepstatin, 1.65 μM antipain, 10.5 μM leupeptin, titrated to pH 7.4 with KOH) which was used for the final step of the endosome preparation. The time course of fluorescence self-quenching was measured.

Flow Cytometry and Sorting

All analysis and sorting was performed on an ATC 3000 (ODAM, BRUKER, Wissembourg, France). The 488 nm wavelength line of an argon ion laser (Innova 90-5, Coherent, Palo Alto, CA) was used for excitation. To increase the sensitivity of the analysis, a cylindrical lens with a 200 mm focal length was introduced in order to narrow the illuminated portion of the sample stream (Gaucher, Grümwald & Frelat, 1988). An interference filter LP 560 (ORIEL, Stratford, CT) placed at an angle of 45° was used for the optic filtering. The wide-angled scattering detector was equipped with a neutral filter (density 2, filter WRATTEN, Rochester, NY) and the fluorescence detector with an OG 515 cut-on filter (Melles Griot, Irvine, CA). Fluorescent beads of 0.9 μm (YG 15702) and 0.25 μm (YG 9834, Polysciences, Warrington, PA) were used to test and calibrate the apparatus. The fluorescence chain gain of the apparatus was calibrated so that the peak of the fluorescence histogram of 0.25 μm beads would appear around channel 90 during all experiments.

The suspension of frog bladder membrane vesicles was diluted in the KCl buffer used at the end of the preparation (100 mM KCl, 5 mM tricine, 1 mM phenyl methyl sulfonide fluoride, 120 μM benzamidine, 1.46 μM pepstatin, 1.65 μM antipain, 10.5 μM leupeptin, titrated to pH 7.4 with KOH) in order to reach a final concentration of 50 to 100 μg of protein/ml. The cytometric analysis was performed at a frequency of 8,000 events per second.

The fraction obtained after sorting was centrifuged at 14,000 × g for 30 min, lyophilized during 2 hr and stored at −80°C for further use.

Electrophoresis

SDS-PAGE experiments were performed with a PhastSystem (Pharmacia, Uppsala, Sweden) (Olsson et al., 1988*a*, *b*). PhastSystem is an integrated system enabling automated separation on small gels (50 × 43 × 0.43 mm) and their development. In our experiments, several types of gel were used: 7.5% homogeneous polyacrylamide gels, polyacrylamide gradient gels from 10 to 15% or from 8 to 25%, and high density gels (all were purchased from Pharmacia). To calibrate SDS-PAGE, several kits were used: the high molecular weight standards (40 to 200 kDa, Bio-Rad, Richmond, CA), the low molecular weight markers (14.5 to 94 kDa, Pharmacia) and the very low molecular weight markers (2,512–16,949 Da, Pharmacia).

Application of samples and electrophoretic procedures were carried out according to the producers manual (File 110, File 111 and File 112). Applicator combs with 12 × 0.3 μl were used. Protein bands were stained according to the high sensitivity silver staining method described in Table 4 of the instruction manual (File 210); except that different developing times (between 2 and 3 min) were used.

The coloration intensity was determined by scanning the gels with an LKB ultrascan (Bromma, Sweden) coupled to a Spectra Physics SP.4100 integrator (Santa Clara, CA) in order to determine the relative proportion of the different revealed proteins.

Results and Discussion

Before proceeding to the isolation of the ADH-induced endocytic vesicles, it was necessary to first be able to correctly label the endosomes and to check whether, under our experimental conditions, the water permeability of these endocytic vesicles was indeed high as shown by Shi and Verkman (1989).

The 6-CF concentration used during the experiments was chosen by analyzing the concentration-dependent self-quenching curve of the probe (*data not shown*). 2 mM of 6-CF were chosen because a slight increase of this concentration led to a great change in fluorescence, a condition which was necessary to perform the stopped-flow experiments.

Then combining the results of different studies, the following experimental conditions seemed the most appropriate to obtain maximal endocytosis of the fluid-phase marker 6-CF: (i) ADH stimulation in the absence of an osmotic gradient increased the water channel density of the apical membrane (Kachadorian et al., 1978; Muller & Kachadorian, 1984), (ii) presence during 5 min of an osmotic gradient activated the endocytosis which was enhanced by the removal of the hormone (Wade et al., 1981; Harris et al., 1986; Coleman et al., 1987). Subsequent steps, rinsing and homogenization, were performed at 4°C to prevent further intracellular processing before epithelial cells were disrupted (Harris et al., 1986; Coleman et al., 1987). Under these conditions we have checked by fluorescence microscopy that 6-CF had indeed been internalized. Intracellular 6-CF was practically undetectable in the cells of the control bladders which had not been submitted to ADH, thus showing that the endocytosis of 6-CF is specific to the action of ADH. This is in agreement with the observations of Harris et al. (1986) and Humbert et al. (1977), who showed that, at rest, toad bladder granular cells internalize fluid phase markers from their apical membrane at an extremely slow rate.

As the labeled endosomes only represent about 5% of the total vesicles (Shi & Verkman, 1989), before measuring water efflux kinetics on the 6-CF-labeled endosomes, we have first checked that we were able to measure water permeabilities on such a minor fluorescent population. In order to mimic the situation, water permeability measurements were performed on a mixture of 6-CF labeled and unlabeled kidney brush border membrane vesicles, known to be highly permeable to water (Pratz, Ripoche & Corman, 1986; Verkman & Ives, 1986; van der Goot et al., 1989). Figure 1*a* shows the kinetics of water efflux obtained by submitting kidney brush-border membrane vesicles among which only

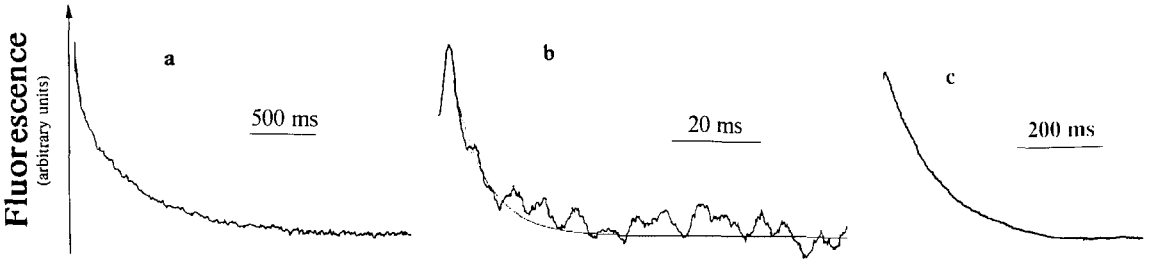

Fig. 1. Time course of fluorescence quenching. (*a*) Osmotic water transport in kidney brush-border membrane vesicles. Kidney brush-border membrane vesicles were prepared as described in "Osmotic Permeability Measurements" in Materials and Methods. 6-CF labeled brush-border membrane vesicles were added to the suspension in order to have 4% of labeled vesicles. The vesicles were submitted to an inwardly directed 100 mOsm mannitol gradient. The induced vesicle shrinkage leads to the quenching of the entrapped 6-CF and a decrease of the fluorescence (T = 22°C). (*b*, *c*) Osmotic water transport in ADH-induced endocytic vesicles from frog urinary bladder. The labeled endosomes were prepared as described in Materials and Methods. 0.1 ml of the frog bladder membrane vesicle suspension containing the 6-CF labeled endosomes was mixed with a hyperosmotic solution in order to reach a 150 mOsm inwardly directed sucrose gradient (T = 22°C). (*b*) The experimental curve as well as the mono-exponential fit is shown. (*c*) The curve was obtained under the same conditions but in the presence of 1 mM $HgCl_2$.

4% were labeled, to an inwardly directed osmotic gradient. The kinetics are in agreement with those previously obtained with kidney brush-border membranes when using light scattering techniques (Verkman & Ives, 1986; van der Goot et al., 1989). Experiments could therefore be performed on frog bladder vesicle suspensions containing 6-CF labeled endosomes. Figure 1*b* illustrates a typical time course of fluorescence quenching obtained by submitting the endosomes to an inwardly directed osmotic gradient. Only the vesicles containing inside fluorescence contribute to the signal. Indeed any extravesicular fluorescence, which might remain at this stage of the experiment although it has essentially been removed by the filtration column, contributes to the overall fluorescence but does not vary with time. The half time ($t_{1/2}$) of fluorescence quenching was 8.23 ± 2.67 msec (n = 6, T = 22°C). The mean diameter of ADH-induced endocytic vesicles in frog urinary bladder epithelial cells, measured by electron microscopy, was 245 ± 13 nm (SEM, n = 25) in agreement with the observations on toad urinary bladder (Harris et al., 1986, 1990; Coleman et al., 1987; Shi & Verkman, 1989; Shi, Brown & Verkman, 1990). Considering this size the measured $t_{1/2}$ corresponds to an osmotic water permeability of approximately 600 μm/sec (estimation calculated using Eq. (4) in van der Goot et al., 1989). This water permeability was inhibited by 90% in the presence of 1 mM $HgCl_2$ (Fig. 1*c*).

These results are in favor of membranes containing water channels (Macey & Farmer, 1970; Solomon et al., 1983; Whittembury et al., 1984; Pratz et al., 1986; van Heeswijk & van Os, 1986; Hoch et al., 1989; Ibarra, Ripoche & Bourguet, 1989; van Hoek et al. 1990; van der Goot et al., 1991) and in agreement with those published by Shi and Verkman (1989) and Wang et al. (1991). Indeed these authors have found that when endocytosis is induced by ADH treatment in the presence of an hypoosmotic lumen solution, the obtained endosomes are essentially composed of vesicles containing water channels. Confident in having labeled the desired vesicles we could proceed with the sorting of the labeled endosomes by flow cytometry. Figure 2*A* shows the fluorescence histogram of the initial suspension of frog bladder membrane vesicles containing the labeled endosomes. In order to check whether the observed fluorescence is significant and can be attributed to the presence of the labeled endosomes, we have also measured the fluorescence of a frog bladder membrane vesicle suspension containing unlabeled endosomes (*see* Materials and Methods and Fig. 2*B*). Both histograms were obtained under identical conditions: the protein concentration of the initial membrane vesicle suspension was 50 to 100 μg/ml and the flow rate was approximately 8,000 vesicles/sec. It appears that the labeled preparation contains a greater amount of highly fluorescent elements (Fig. 2*A*) which are observed in the fluorescence channels above channel 40. This channel was thus chosen as threshold. This window contained 1–1.5% of fluorescent vesicles. This percentage is slightly lower than the percentage of labeled vesicles in the homogenate (around 5% in toad bladder according to Shi and Verkman, 1989) but this is because we have imposed a threshold in size and in fluorescence. In order not to miss any of the desired elements during sorting, for each identified fluorescent vesicle, 3 drops (the drop containing the endosome, the drop before and the drop after) were deviated from the main droplet flow jet

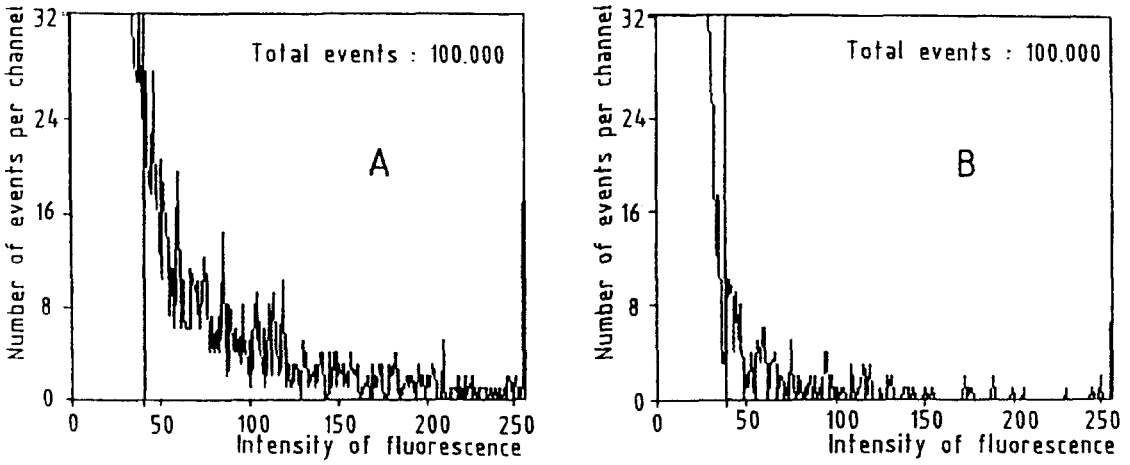

Fig. 2. Flow cytometry fluorescence histograms of the suspension of frog bladder membrane vesicles containing labeled (*A*) or unlabeled (*B*) endosomes. The two histograms result from the analysis of the same number of events. In *A*, a labeled population appears beyond channel 40 which is not observed in *B* (the scale is identical for both figures).

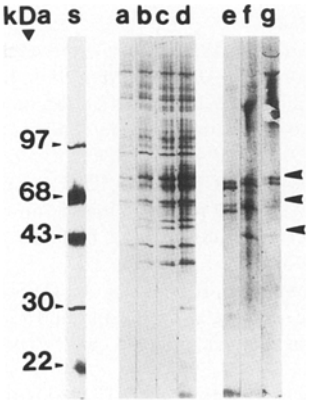

Fig. 3. SDS gel analysis of ADH-induced endocytic vesicles sorted by flow cytometry (*e, f, g*) and of a suspension of frog urinary bladder membrane vesicles (*a, b, c, d*). (*a–d*) Protein content of a suspension of frog urinary bladder membrane vesicles: 300 μl of SDS solubilizing buffer containing Tris-HCl (pH 7.5), 10% glycerol, 0.05% bromophenol blue, 10 mM dithiothreitol were added to 200 μl of frog bladder membrane vesicle suspension. After 5 min incubation at 95°C, samples were centrifuged at 14,000 × *g* for 5 min. The supernatant was analyzed on an SDS 10–15% polyacrylamide gradient gel. Increasing amounts of protein (12 to 60 ng) were deposited from *a* to *d*. (*e–g*) ADH-induced endocytic vesicles sorted by flow cytometry: 10 μl of SDS solubilizing buffer was added to the bottom of the tube containing the lyophilized endosome sample and then the same protocol as for *a–d* was applied. *f* and *g* were obtained with the same endosome preparation. It was estimated that 2.4 and 4.3 ng of protein were deposited on *g* and *f*, respectively. *e* was obtained with another sample. It was estimated that 7.3 ng were deposited. *s*: molecular weight markers.

into a separate tube (Pinkel & Stovel, 1985). As each drop surrounding the labeled drop either contains an endosome, an undesired vesicle or is empty, our method of sorting inevitably leads to a certain contamination.[1]

But the quantity of recuperated endosomes remains very small. To make a rough approximation of the protein content let us assume that there is a linear relationship between weight amount of protein and number of vesicles. Then considering the protein concentration of the initial sample (50 to 100 μg of protein/ml) and the fact that about 1 ml is sorted in 5 hr, the recovered fraction (1–1.5%) would correspond to about 0.5 to 1.5 μg. The PhastSystem was therefore chosen for the electrophoretic analysis: small samples (0.3 μl) are disposed on the gels and less than 1 ng/protein can be revealed using a high sensitivity silver staining method.

The analysis on 10–15% SDS polyacrylamide gels of the membrane fractions before and after sorting are displayed in Fig. 3. The protein pattern obtained with the sorted endosomes (Fig. 3*a*) is considerably different from that obtained from the initial vesicle suspension (Fig. 3*b*). In the endosomes, the major proteins revealed by the silver

[1] This contamination can roughly be estimated. The probability that a drop contains a vesicle, fluorescent or not, is of 0.2, considering that 8,000 events are analyzed per second at 40 kHz droplet frequency. As three drops are deviated each time, it means that, for every fluorescent endosome, 2 × 0.2 undesired elements are also sorted. This approximated calculation lead to a 29% contamination (expressed in number of selected vesicles).

staining method were two bands at 42 and 55, a double band at 71 kDa, as well as a group of proteins clustered from 120 to 170 kDa which might be due to aggregation during the sample heating. As determined by scanning the gels, the 43, 55 and 71 kDa proteins represent 10, 38 and 31%, respectively, of the total protein of the sorted fraction whereas they only represent 1, 4 and 2%, respectively, of the protein of the initial vesicle suspension.

While proteins with the same molecular weight have been previously observed by Harris et al. (1987) in a purified endosome fraction obtained by the density shift maneuver, these authors also found several low molecular weight proteins (mainly 7, 15, and 17 kDa), which were apparently absent in our preparation. Indeed by carrying out the electrophoresis on high density gels as well as on gels having a polyacrylamide gradient of 8–25%, we could not detect any proteins with an apparent molecular weight of less than 40 kDa in our endosome preparation. We cannot, however, exclude the possibility that other proteins may be present that cannot be identified with our method, either because they are present in too small quantities or because they cannot be revealed by silver staining.

The present study shows that flow cytometry and sorting is a powerful technique to isolate ADH-induced endocytic vesicles. This was recently confirmed, by data reported in abstract form, by Grossman et al. (1991). Under our experimental conditions, three major proteins of 43, 55 and 71 kDa are found in the sorted endocytic vesicles. These are likely to be involved in the mechanisms of water transport across the apical membrane of frog urinary bladder under ADH stimulation. It would be interesting, however, to improve the method by performing a partial purification using differential centrifugation as described by Harris et al. (1990) prior to the sorting. The endosome concentration before sorting would be increased, the number of vesicles analyzed per second could thus be reduced, and the final contamination would be greatly diminished. The characterization of the obtained sample can lead to a more precise identification of the proteins involved in the membrane water permeabilization.

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