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Rapid Swelling of a CHO-K1 Aspartate/Glutamate Transport Mutant in Hypo-osmotic Medium

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Abstract. Two Chinese hamster ovary cell (CHO-K1) mutants selected for defective glutamate transport via system X_{AG}^- are also highly permeable to small neutral molecules. Light microscopy demonstrated that exposure of one of these mutants, Ed-A1, to hypo-osmotic medium led to extremely rapid swelling, presumably due to increased water flux. When placed in 20% saline, Ed-A1 cells swelled to three times their original volume within 15 sec, a sixfold larger increase than parental CHO-K1. In spite of this rapid volume increase, mutant and wild-type cells remained viable for 20 min in dilute saline. A regulatory volume decrease in Ed-A1, and the continual swelling of CHO-K1, resulted in the two cells achieving equal size after 5 min in 20% saline.

The time course of these volume changes permitted analysis of large numbers of cells by a hydrodynamic technique, steric field flow fractionation (FFF). Steric FFF demonstrated the expected inhibition of osmotic swelling of human erythrocytes by the mercurial, *p*-chloromercuribenzenesulfonic acid (PCMBS). However, PCMBS increased the apparent swelling rate of Ed-A1 and CHO-K1, suggesting that an aquaporin-like molecule is not responsible for any significant fraction of the water fluxes into either line. PCMBS also strongly inhibited aspartate transport by system X_{AG}^-. By taking advantage of their different swelling rates in hypotonic medium, steric FFF can separate mixtures of CHO-K1 and Ed-A1.

Key words: System X_{AG}^- — CHO-K1 mutant — Osmotic shock — Volume — Steric field-flow fractionation — PCMBS

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Introduction

We have recently discovered a novel membrane perturbation of two CHO-K1 amino acid transport mutants. The mutants, Ed-A1 and Ed-B8, were selected for defects in amino acid transport system X_{AG}^- by a suicide protocol using [^{3}H]glutamate (Ash et al., 1993). System X_{AG}^- catalyzes sodium-dependent import of aspartate and glutamate and the mutations in Ed-A1 and Ed-B8 produced significant alterations in kinetic parameters of this system (Ash et al., 1993). Subsequent characterizations of these and additional X_{AG}^- mutants revealed that Ed-A1 and Ed-B8 suffered an additional alteration: an increase in their permeability to small neutral molecules (Igo & Ash, 1996). This was noticed when a standard technique for measuring cell volume, permeation by [^{14}C]urea (Vasquez, Ishibashi & Howards, 1982; DeBusk & Ash, 1993), did not work, apparently because urea was lost from the mutants extremely quickly when they were rinsed. These mutants also proved to be more permeable than CHO-K1 to glycerol and were seen to swell rapidly when placed in diluted media (Igo & Ash, 1996). However, cytoplasmic sodium levels are normal and amino acid pools are high in the mutants (Ash et al., 1993), indicating that their plasma membranes are not generally leaky. Other mutants with more severe defects in X_{AG}^- activity did not share this high permeability (Igo & Ash, 1996).

We think it is likely that single mutations have simultaneously rendered the X_{AG}^- protein defective for amino acid transport and created the membrane leak for urea, glycerol, and water in Ed-A1 and Ed-B8. Alternatively, these mutants could be expressing additional new components that facilitate transmembrane movement of small neutral molecules, such as aquaporins (Engel, Walz & Agre, 1994; Agre, Brown & Nielsen, 1995). Testing the first hypothesis will eventually require clon-

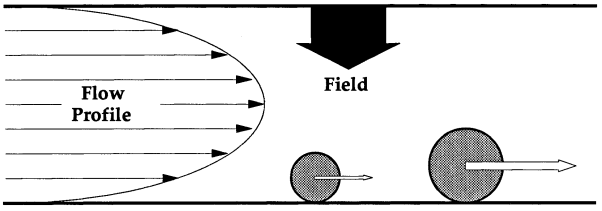

Fig. 1. Schematic representation of separation in a steric FFF channel. Two particles of different sizes are held against one wall by an applied field while being eluted by fluid flowing along the channel. Due to friction at the walls, fluid flows faster in the center of the channel. Therefore, the larger particle moves faster, and will be eluted sooner, because it is exposed to faster fluid flow.

ing the wild-type and mutant X^-_{AG} genes. Alternatively, genes responsible for the high permeability phenotype might be identified and cloned by virtue of their ability to induce rapid swelling and then tested in an expression system to see if they confer system X^-_{AG} activity. This would require developing a procedure for separating cells based on different swelling rates. While pursuing this goal, we found that an effective separation procedure could be employed immediately as an analytical tool, allowing us to test the aquaporin-expression hypothesis.

The nature of the phenomenon of interest here, short-term differences in swelling rates, appeared to exclude direct applications of familiar separation techniques such as velocity and density gradient centrifugation, gel filtration, electrophoresis, or two-phase polymer partitioning. To achieve a rapid and effective discrimination between Ed-A1 and CHO-K1 we turned to size-selective steric field-flow fractionation (FFF). FFF is a family of analytical separation techniques applicable to macromolecules and particles over a 10^{15}-fold mass range that corresponds to a 10^5-fold diameter range (Giddings, 1993). Steric FFF (Caldwell et al., 1984) provides rapid separation of particles in the 1–100 μm diameter range, which is clearly useful for cellular analyses. This technique is based on elution from a thin ribbonlike laminar flow channel. Differential transportation of particles in the channel is induced and controlled by the flow rate in combination with a field applied perpendicular to the flow. Here, we used a centrifugal (C-FFF) field. As illustrated in Fig. 1, separation in steric FFF occurs because larger particles, held against one wall by the field, protrude further into faster flowing fluid and elute earlier than smaller particles. Steric FFF separation of latex particles and silica beads has been very successful (Giddings, 1993). In other applications, cells have been shown to separate according to shape, density, carrier fluid viscosity (*unpublished data*), and mechanical properties of the cell membrane, such as deformability (Tong & Caldwell, 1995). Besides its useful size range, the principal advantages steric FFF provided here were its speed and simplicity. The swelling differential lasts only minutes, making a rapid procedure essential.

Materials and Methods

Biochemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted; other chemicals were reagent grade or better.

Cells and Culture Conditions

CHO-K1 (Chinese hamster ovary, subclone K1) cells were obtained from the American Type Culture Collection (CCL 61, Rockville, MD). Ed-A1 cells are a clonal derivative of CHO-K1, obtained from an ethylmethane sulfonate mutagenized stock by tritium-suicide selection for reduced glutamate transport (Ash et al., 1993). Cells were grown as previously described (Ash et al., 1993) at 37°C in a humid 5% CO_2-95% air atmosphere in D/F H5C5 medium: 45% Dulbecco modified Eagle medium with nonessential amino acids, 45% Ham F12 medium, 5% horse serum, 5% calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were removed from dishes for passage every three to four days by rinsing with HBS (137 mM NaCl, 0.7 mM K_2HPO_4, 10 mM HEPES (Research Organics, Cleveland, OH), pH adjusted to 7.4) and incubation in a thin film of 0.01% (w/v) trypsin in HBS. This resulted in a homogeneous cell suspension, typically with >98% single cells. When necessary, cells were counted using a Coulter model ZM cell counter (Coulter Electronics, Hialeah, FL). For microscopic and FFF analyses of swelling, cells were grown for two days before trypsinization and counting. They were then pelleted by centrifugation at 700 rpm for 5 min in a clinical centrifuge and resuspended at 4×10^6/ml in culture medium. For some studies, cells were loaded with a stable cytoplasmic fluorescent marker by incubating two-day-old-cultures on dishes for 10 min at 37°C in 10 μM 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes, Eugene, OR) before rinsing and trypsinization. This was accomplished by removing an aliquot of the culture medium to a tube, adding an appropriate volume of 5 mM CMFDA in DMSO, mixing, and returning the medium to the dish. Fluorescent label in cells was observed with a Zeiss IM 35 Photomicroscope using epifluorescence illumination and specific interference filters for fluorescein.

Blood was collected from healthy laboratory personnel into EDTA-containing tubes (Becton-Dickinson, Rutherford, NJ). The red cells were washed by centrifugation three times in isotonic phosphate buffered saline (PBS) solution (0.01 M phosphate, 0.137 M NaCl, 0.0027 M KCl, pH = 7.4) to remove blood plasma, white blood cells, and platelets.

Direct Observation of Cell Swelling

To initiate a timed swelling assay, room temperature distilled water in a transfer pipette was added to a cell suspension placed in the tip of a small polystyrene tube. The diluted cell suspension was immediately picked up with the same pippette and transferred onto a glass coverslip mounted underneath a 5-mm round hole in a 35-mm culture dish, and another coverslip was placed inside the dish, atop the liquid. Photographs were taken on 35 mm film using a 40× objective and Hoffman Modulation Contrast optics on a Nikon Diaphot photomicroscope, beginning as soon as possible and *ca.* 15 sec after the initial dilution. Images on photographic negatives were converted to digital format by a Leafscan 45 film scanner (Leaf Systems, Southboro, MA) and stored as PICT files. The nearly spherical cell outlines were traced using NIH image 1.57 software for the Macintosh, which reported major (*a*) and minor (*b*) axes in microns. The volume of a cell was then estimated by rotating its elliptical representation around the major axis:

$$Volume\ (pl) = \frac{4/3\pi ab^2}{8000}. \quad (1)$$

Steric FFF Analysis

The steric centrifugal FFF (C-FFF) channel was curved to fit inside the rotor basket of a centrifuge, so that the channel encircled the centrifuge axis like a belt. This setup has been described in detail elsewhere (Caldwell et al., 1984). The walls consisted of a polished corrosion-resistant alloy, Hastelloy C, and its dimensions were 0.0254 cm × 2.0 cm × 94.5 cm. The rotor radius was 15.5 cm, and the *g*-force acting on the cells as a function of selected *RPM* (centrifugal spin rate in rotations per min) was calculated as:

$$g(m \cdot s^{-2}) = 0.155 \times \left(\frac{2\pi RPM}{60}\right)^2. \quad (2)$$

Carrier medium was fed to the channel by a peristaltic pump (Rainin Instruments, Woburn, MA). The channel effluent was monitored with a Linear 106-UV detector (Linear Instruments, Reno, NV) operating with a 254 nm light source. The detector signal was displayed and times associated with elution peak positions determined using a computer program developed in our laboratory. Sample fractions were collected at the detector outlet for purity or viability analysis.

Samples of cells or Polystyrene beads were injected by a syringe needle into the channel through a septum injection port mounted on one of the walls. Injection sizes were between 1–10 μl with cell concentrations *ca.* 4×10^6/ml. The carrier medium for cells was PBS containing 0.1% bovine serum albumin (BSA) or a distilled water dilution of PBS containing 0.1% BSA. The medium used for analyzing Polystyrene beads contained 0.1% of the detergent FL-70 (Fisher Chemical, Fair Lawn, JN) in deionized water. After injection, the flow was stopped for 5 min to allow cells to settle fully (or be relaxed) onto the outer channel wall. This relaxation process is important for the cells to reach their equilibrium position in the channel during separation, and the relaxation time required for different samples depends on the cell size, channel thickness, carrier fluid viscosity, sedimentation field as well as on the density difference between cell and carrier fluid. After the stopped-flow relaxation period, the pump was activated and carrier fluid fed through the channel at a controlled rate.

Aspartate Transport Measurement

The activity of system X^-_{AG} was estimated by measuring the 2 min uptake of 1–1000 μM [^{3}H]aspartate (10 μCi/ml, Amersham, Arlington Heights, IL) from HBS · MCD (HBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 g D-glucose/liter) at 37°C by cells cultured for 24 hr in 24-well cluster trays as previously described (Ash et al., 1993; Igo & Ash, 1996). For these experiments, cells were preincubated for 10 min at room temperature with 0.5 ml HBS ± 2 mM *p*-chloromercuribenzenesulfonic acid (PCMBS) before rinsing and exposure to [^{3}H]aspartate. Velocity data were fit to kinetic models using the Marquardt least-squares method. The goodness-of-fit probability, *q*, was calculated from the χ^2 statistic associated with the fitted parameters and fits with $q > 0.1$ were judged acceptable (Ash & Igo, 1993; Ash et al., 1993).

Results

Swelling Kinetics of CHO-K1 and Ed-A1

Previous, nonquantitative observations indicated that differences in swelling rates between CHO-K1 and mutants

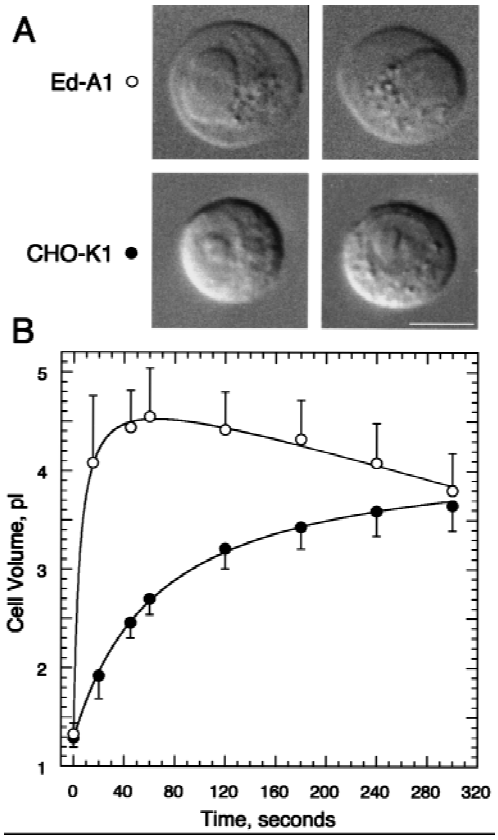

Fig. 2. Microscopic observation of swelling of CHO-K1 and Ed-A1 cells in 20% medium. (*A*) Photomicrographs of two cells of each line taken 45 sec after medium was diluted to 20% with water. The white bar indicates 10 μm. (*B*) Average cell volumes (CHO-K1 ●, Ed-A1 ○) were estimated from planar measurements of ten cells of each line followed for 5 min after the medium was diluted. The earliest time point, 15 sec for Ed-A1 and 20 sec for CHO-K1, includes only five of the ten cells for each, as the other five had not settled sufficiently to permit measurement. A different set of ten cells in undiluted medium was used to determine the 0-sec value. Error bars represent 1 SEM. Data were fit to a model combining a hyperbolic and a linear term, as in Fig. 4.

Ed-A1 and Ed-B8 could be detected when the cell lines were abruptly exposed to dilute media (Igo & Ash, 1996). For convenience, we decided to limit studies here to the parental line and one mutant, Ed-A1. The effect on viability of exposure to PBS concentrations ranging from 100% to 20% was determined for both cell lines by measuring colony formation after a 20-min saline incubation. Plating efficiencies were reduced from *ca.* 60% to 50% by a 20-min exposure to 100% PBS in culture tubes rotated to keep cells suspended, but dilution of the saline down to 20% had no additional effect.

Photomicroscopic observations of the response of CHO-K1 and Ed-A1 cells to a 45-sec exposure to 20% culture medium are shown in Fig. 2*A*. It is obvious that Ed-A1 cells had swelled more quickly than wild-type CHO-K1. In the mutants at 45 sec, nucleoli have be-

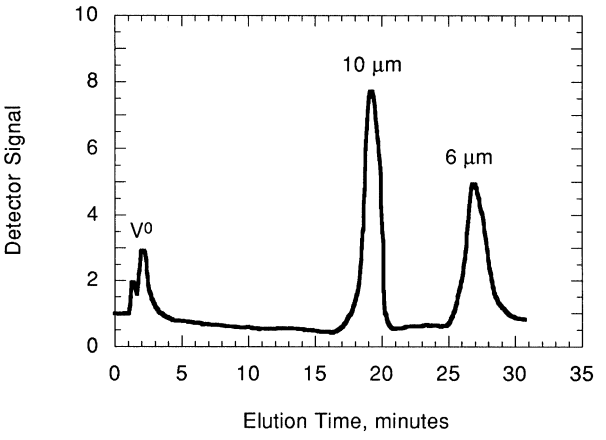

Fig. 3. Elution profile of a separation of 10 μm and 6 μm diameter Polystyrene latex beads by C-FFF. The V° peak represents unretained materials introduced by sample injection. Elution conditions used: spin rate = 180 *RPM*, yielding field strength of 6 g, flow rate = 2.35 ml/min.

come indistinct and the nucleus with associated cytoplasm was surrounded by a clear zone underlying the plasma membrane. Nucleoli in CHO-K1 required several min in 20% PBS to disappear and no large clearing under the plasma membrane was observed at any time. Measurements of cells, followed for 5 min in 20% medium, are shown in Fig. 2*B*. The two cell types did not differ in size initially, but Ed-A1 cells were nearly fully swollen at the earliest time point, a 3-fold volume increase at 15 sec, while CHO-K1 volume had increased by 50% at 20 sec. This volume differential diminished upon extended incubation in the hypotonic medium, with CHO-K1 continuing to swell and Ed-A1 gradually shrinking after 60 sec so that average volumes of the two cell types did not differ significantly at 5 min. It is likely that the shrinking of Ed-A1 represented a regulatory volume decrease (Chamberlin & Strange, 1989), rapidly stimulated by its abrupt swelling. When swollen Ed-A1 cells are returned to iso-osmotic medium, they abruptly shrink (*results not shown*).

Size Analysis by Steric C-FFF

As an example of a simple size-based separation, Fig. 3 shows the elution profile of a mixture of 6 μm and 10 μm diameter Polystyrene latex particles analyzed by steric C-FFF. A complete, baseline separation of these rigid, uniform particles was possible using an arbitrarily selected sedimentation force and carrier flow rate. When analyzing living cells, however, several preliminary experiments were required to establish optimal separation conditions.

The elution behavior of cells suspended in isotonic and hypotonic media and analyzed by steric C-FFF is

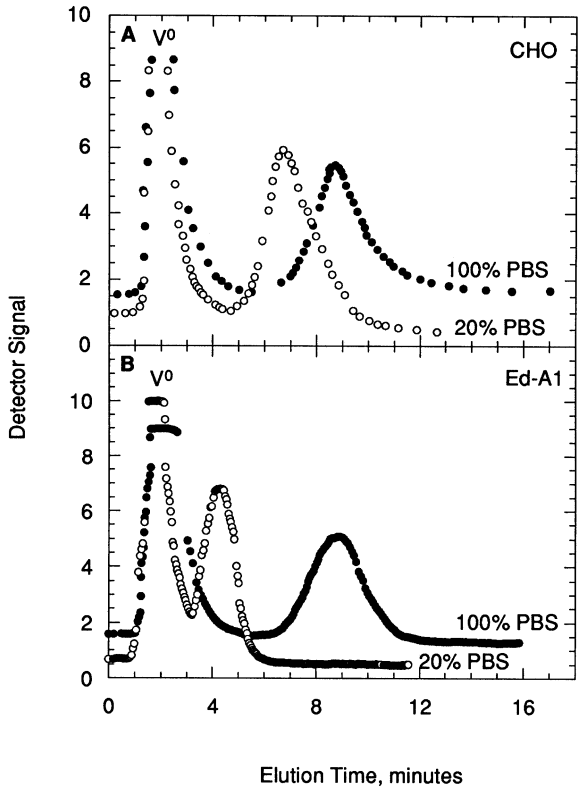

Fig. 4. C-FFF analysis of cells eluted with 100% or 20% PBS. Cells of one line were introduced into the channel and relaxed in 100% PBS for 5 min. The results for elution with each saline concentration are indicated in (*A*) for CHO-K1 and (*B*) for Ed-A1. Some components of the cell culture medium, such as serum proteins and nucleotides, absorb strongly at 254 nm and are responsible for the large peak of unretained material, labeled V°. Elution conditions used: spin rate = 150 *RPM*, field strength = 4 g, flow rate = 3.0 ml/min.

demonstrated in Fig. 4. In Fig. 4*A*, a sample of CHO-K1 cells was injected and relaxed in isotonic (100%) PBS buffer for 5 min. Buffers of 100% PBS or 20% PBS were then introduced as carrier fluid in each of two separate runs to examine the elution behavior of CHO-K1 cells under normal and hypotonic conditions. As expected, the swollen CHO-K1 cells carried by 20% PBS eluted faster than the cells carried by 100% PBS. Fig. 4*B* illustrates the result of an identical protocol applied to Ed-A1 cells. By comparing Fig. 4*A* and *B*, one can see that both the parental and mutant cells eluted at nearly the same time when they were carried by 100% PBS (8.80 min for Ed-A1 and 8.70 min for CHO-K1). However, Ed-A1 cells eluted at a faster rate (4.35 min for Ed-A1 and 6.70 min for CHO-K1) when they were carried by 20% PBS. This was assumed to be due to the different initial swelling rates of the two cell types seen in Fig. 2. Elution of both cell types under the same conditions were repeated 10 times, and the range of elution times were within ±2.0% of the average elution time.

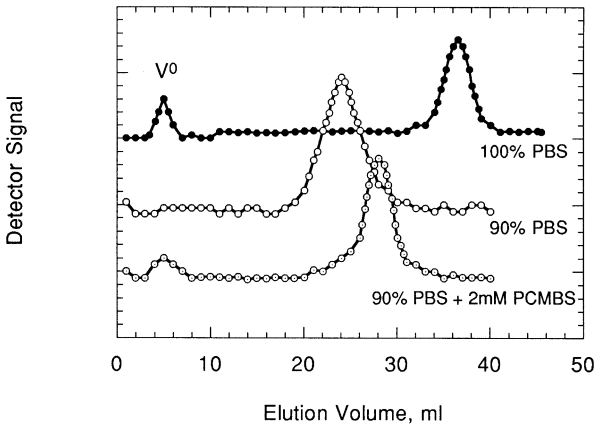

Fig. 5. C-FFF analysis of the effect of PCMBS on elution of human red blood cells in dilute saline. As indicated on the figure, cells initially relaxed in 100% PBS were eluted with 100% PBS, 90% PBS, or treated with 2 mM PCMBS for 30 min before injection and elution with 90% PBS. Elution conditions used: spin rate = 150 *RPM*, field strength = 4 g, flow rate = 3.0 ml/min.

Effects of PCMBS on Swelling and Transport Activity

As a test of the hypothesis that abnormal expression of an aquaporin is responsible for the rapid volume increases seen in Ed-A1, effects of PCMBS on cell swelling were investigated, taking advantage of the ability of steric FFF to analyze cell swelling, as seen in Fig. 4. This compound is known to slow the swelling of erythrocytes by inhibiting aquaporin-mediated water permeability (Macey & Farmer, 1970; Macey & Karan, 1993). Human red blood cells in several dilutions of PBS were examined using steric C-FFF to establish conditions which maximized effects of PCMBS on elution behavior. As demonstrated in Fig. 5, peak elution times were significantly decreased when cells were eluted with PBS diluted to 90%. Pretreatment of the cells with 2 mM PCMBS for 30 min at room temperature significantly increased elution times of cells in 90% PBS. Thus, the inhibition of water entry into human red blood cells by PCMBS, which will slow their volume increase and delay elution times, could be detected by steric C-FFF.

In contrast, no increase in peak elution times was detected when either Ed-A1 or CHO-K1 were pretreated with 2 mM PCMBS and then eluted, as in the experiments of Fig. 4, with 20% PBS (*data not shown*). In fact, it appeared that treating these cells with PCMBS decreased their elution times, although the effect was difficult to measure accurately because the swollen cells eluted very close to the flow-through, V^o, peak. To investigate this further, elutions were made with a series of less diluted saline solutions, up to 50% PBS, and, as expected, peak elution times increased as the concentration of the diluted elution fluid was increased. As seen in

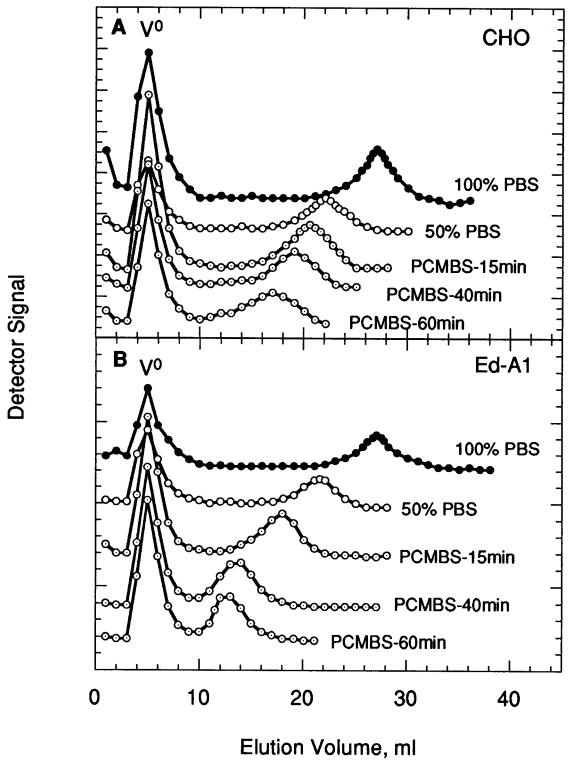

Fig. 6. C-FFF analysis of the effect of PCMBS on elution of CHO and Ed-A1 in dilute saline. The results of elution with 100% and 50% PBS and treatment with 2 mM PCMBS for three time periods before injection and elution in 50% PBS are shown in (*A*) for CHO-K1 and (*B*) for Ed-A1. Elution conditions used: spin rate = 150 *RPM*, field strength = 4 g, flow rate = 3.0 ml/min.

Fig. 6, elution with 50% PBS still resulted in a detectable decrease in peak elution times, compared to elution with 100% PBS, however, with the difference observed between CHO-K1 and Ed-B8 was very small in this case. When cells were pretreated with PCMBS and eluted with 50% PBS, the elution times of both CHO-K1 (Fig. 6*A*) and Ed-A1 (Fig. 6*B*) were significantly decreased. Figure 6 also shows that the longer the pretreatment by PCMBS, the greater the decrease in elution times in 50% PBS. Thus, PCMBS did not reduce swelling in either cell line, but rather increased the swelling effect of dilute medium, with the rate of increase greater in Ed-A1. Pretreating cells with PCMBS for 60 min did not effect their elution in isotonic PBS (*data not shown*).

The effect of PCMBS on the activity of system X^-_{AG} of Ed-A1 was determined by measuring [^{3}H]aspartate uptake, as shown in Fig. 7. High affinity, saturable uptake of aspartate is here assumed to occur solely via system X^-_{AG}. The hyperbolic uptake profile seen for untreated cells demonstrates the partial activity of system X^-_{AG} in this mutant: V_{max} for CHO-K1 is *ca.* 4.5-fold higher when measured in cells immediately removed from culture medium (Ash et al., 1993). When the data

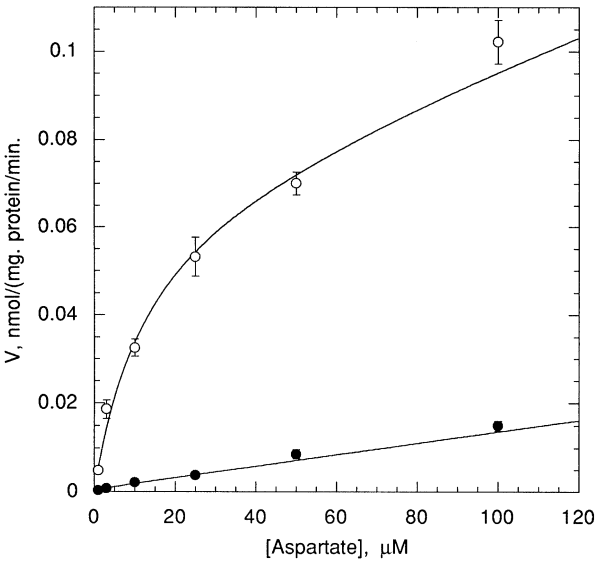

Fig. 7. Effect of PCMBS on aspartate uptake by Ed-A1. Cells were exposed for 10 min at room temperature to HBS (○) or HBS containing 2 mM PCMBS (●) before determining aspartate uptake velocities. The average of three velocity values is shown ±1 SD when larger than the symbols and were fit with $V = V_{max}[S]/(K_m + S) + k_D[S]$. Data for [Aspartate] ≤100 μM are shown to emphasize the saturable component of uptake, which is attributed to system X_{AG}^-. The parameters (±1 SD) for cells exposed to HBS alone were: $V_{max} = 0.069 \pm 0.005$ nmol/mg protein/min, $K_m = 13 \pm$ μM, $k_D = (3.3 \pm 0.2) \times 10^{-4}$ min^{-1}, and goodness-of-fit, $q = 0.15$. PCMBS severely affected all parameters, as discussed in the text.

of Fig. 7 are judged by eye, PCMBS appears to eliminate saturable (hyperbolic) aspartate uptake, however, the goodness-of-fit probability for a linear model, $V = k_D[S]$, was $q < 10^{-5}$, while for a model combining the Michaelis-Menten equation with a nonsaturable term, $V = V_{max}[S]/(K_m + S + k_D[S]$, the probability was $q = 0.39$. In Ed-A1 cells treated with PCMBS, V_{max} was reduced by 99%, K_m by 60%, and nonsaturable uptake, k_D, by 62%. Inhibition of system X_{AG}^- by 2 mM PCMBS in CHO-K1 was verified at 1 μM aspartate, where uptake is dominated by system X_{AG}^-. PCMBS treatment reduced total uptake of 1 μM aspartate by 92%, compared to 94% inhibition in Ed-A1 at this concentration, demonstrating that PCMBS severely inhibited system X_{AG}^--mediated activity in the parental cells, as well.

Separation of Ed-A1 from CHO-K1 by FFF

The difference in peak elution times observed between CHO-K1 and Ed-A1 eluted with 20% PBS in Fig. 4 suggested that mixtures of these cells could be resolved by steric C-FFF. The elution behavior of mixtures of the two cell types, equilibrated and eluted with either 100% or 20% PBS, is seen in Fig. 8. The channel was prefilled with 100% isotonic PBS buffer, and a 1:1 mixture was

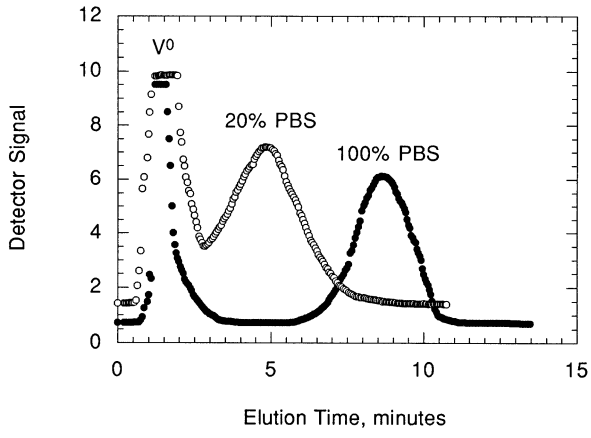

Fig. 8. C-FFF analysis of 1:1 mixtures of CHO-K1 and Ed-A1 equilibrated and eluted in 100% or 20% PBS. As indicated by the peaks, one mixture was relaxed in 100% PBS for 5 min and carried through the channel with same buffer. The other mixture was relaxed in 20% PBS for 30 min and carried through the channel without changing buffer. Elution conditions were as stated in Fig. 4.

injected into and carried through the channel without a change in buffer. One peak was observed, indicating that the two cell types are of equivalent size in an isotonic environment. In a subsequent step, the channel was prefilled with 20% PBS and the mixture was injected into the channel and incubated for 30 min before elution with the same saline. In this case, as well, there was no evidence of separation.

Having shown that CHO-K1 and Ed-A1 cells behave similarly and are not separable by steric C-FFF when equilibrated with either isotonic or hypotonic saline, the possibility of separating the two cell types during their transient swelling stage was explored. Ed-A1 cells were labeled with CMFDA to permit their subsequent identification by fluorescence microscopy. A 1:1 mixture of labeled Ed-A1 and unlabeled CHO-K1 was injected and allowed to relax in the channel filled with 100% PBS. At the end of the stop flow period, 20% PBS was introduced to carry the mixture through the channel. As shown in Fig. 9, two peaks were observed with elution times corresponding to those of CHO-K1 and Ed-A1 cells examined individually under similar conditions (Fig. 4). To verify that the faster eluting peak consisted of Ed-A1 cells, fractions were collected at three positions of the elution profile and samples examined by fluorescence microscopy to quantitate contamination by unlabeled CHO-K1 cells. The three fractions covering the fast eluting peak all contained >97% fluorescently labeled cells. Companion experiments using CMFDA labeled CHO-K1 cells, or unlabeled cells of both lines, showed similar results (*data not shown*). Samples of the eluted fractions were also placed in culture medium and incubated for several days. Both Ed-A1 and CHO-K1 cells survived the swelling and centrifugations: cells can

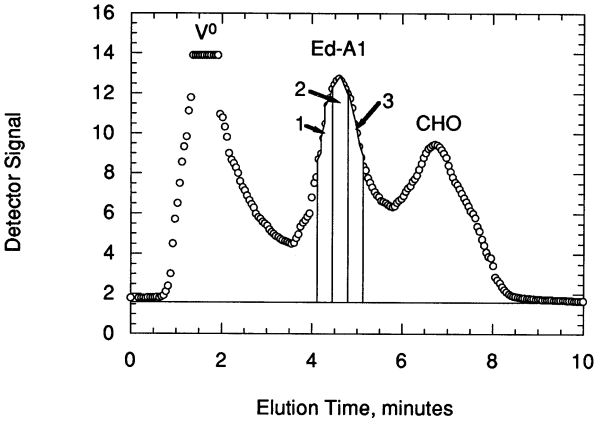

Fig. 9. Separation of a mixture of CHO-K1 and Ed-A1 by C-FFF. A 1:1 mixture of CHO-K1 and CMFDA-labeled Ed-A1 cells were relaxed in 100% PBS for 5 min, then carried through the channel with 20% PBS. Fractions of the fast eluting peak were collected from three positions as indicated on the plot and were analyzed by fluorescence microscopy. Fields were scored until >150 cells had been counted. The purities, calculated as 100 × (fluorescent cells/total cells), were: fraction 1, >99%, fraction 2 >98%, fraction 3 >97%. Elution conditions used: spin rate = 150 *RPM*, field strength = 4 g, flow rate = 3.1 ml/min.

attach, divide, and form colonies. Absolute recovery efficiencies have not yet been determined.

Discussion

Osmotic Sensitivity of Ed-A1

The rapid swelling of Ed-A1 in hypotonic medium is striking. The threefold volume increase observed in these cells at 15 sec in 20% PBS would require a twofold increase in surface area if they were simple spheres. The photomicroscopic method used here to monitor size changes missed events occurring before 15 sec, and the actual swelling rates are likely to be higher than could be estimated from the data of Fig. 2. The plasma membranes of these cells are capable of prompt, impressive reorganization. As pointed out by Morris and coworkers (Reuzeau et al., 1995; Wan, Harris & Morris, 1995), who studied osmotic responses of molluscan neurons, the conventional understanding of cell swelling is largely based on analyses of erythrocytes, which have only a limited capacity to expand before bursting. The neurons studied by Morris's group appeared to use internal membrane stores and cytoskeletal machinery to withstand severe osmotic stresses. Ed-A1 and CHO-K1 are likely to employ similar survival strategies, although the faster response of hamster cells to hypo-osmotic stress suggests significant differences in water permeability and/or in mechanisms involved in resisting swelling in these cells. It took the molluscan neurons at least 20 min to increase their relative volume threefold when placed in distilled water (Wan et al., 1995); Ed-A1 achieved this increase in 1/80th of that time in medium diluted fivefold.

The rapid swelling of Ed-A1 is also unusual: we are not aware of reports of a similar permeability change induced by mutation. Harris described the selection of Chinese hamster V79 cells able to grow in hypertonic media (Harris, 1993), but did not report how these cells responded to acute changes in osmolarity.

Why Does Ed-A1 Swell So Quickly?

It is possible that changes outside the plasma membrane in the mutant cells, for instance in cytoskeletal elements, could be responsible for their increased osmotic sensitivity. However, the fact that these cells are known to be more permeable to urea and glycerol (Igo & Ash, 1996) suggests that altered transmembrane water entry is the most likely cause of the increaed swelling rate of Ed-A1. Two extreme explanations for such an alteration were suggested above. It could result from a change in the system X^-_{AG} transporter produced by the mutation that created the amino acid transport defect, or it could result from a second, independent event, such as the expression of an aquaporin. Aquaporins are integral membrane proteins that facilitate transmembrane water movements in a variety of cells, from erythrocytes to kidney collecting tubule elements (Agre et al., 1995; Engel et al., 1994). Aquaporins appear to provide the dominant site of transmembrane water flux in cells that express them, although other solute transporters may contribute to water flow (Verkman, 1992). Artificial expression of an aquaporin in frog oöcytes produced rapid swelling compared to control eggs when both were placed in hypotonic media (Preston et al., 1992). Therefore, one could reasonably consider the possibility that, for reasons unrelated to the selection against system X^-_{AG}, the cell which founded the Ed-A1 clone happened to express an aquaporin. We tested this by use of an inhibitor. Water movement through aquaporins is sensitive to inhibition by sulfhydryl reagents such as mercury ions and the organic mercurial, PCMBS (Knepper, 1994; Macey & Farmer, 1970; Preston et al., 1992). C-FFF analysis of red cells treated with PCMBS demonstrated an expected increase in elution time in hypotonic saline, consistent with an inhibition of swelling. However, this agent reduced the retention times of both Ed-A1 and CHO-K1 and, therefore, appeared to increase their swelling rates. Such an increase in swelling produced by PCMBS was unexpected and strongly suggests that a typical aquaporin is not mediating a significant fraction of transmembrane water flow in either cell line. Since PCMBS did not change the elution behavior of CHO and Ed-A1 in isotonic PBS, we could exclude the possibility that this unexpected behav-

ior was due to other effects of PCMBS, such as changes in membrane deformability, in cell density, or by induction of aggregation.

Explaining the behavior of Ed-A1 by novel expression of an aquaporin-like molecule seems unlikely for another reason: increased permeability and rapid swelling were observed in both Ed-A1 and Ed-B8 (Igo & Ash, 1996). These clones arose from the same mutagenized population, but they originated from separate selection dishes and have different alterations in X^-_{AG} activity (Ash et al., 1993). They, thus, appear to be independent mutants. Random expression of aquaporins in CHO-K1, if it occurs at all, must be rare as these cells swell slowly, and it is unlikely that two aquaporin-expressing clones would be picked at random. Since system X^-_{AG} was the target of the selection that produced these two mutants, it seems reasonable to propose that alterations of X^-_{AG} are responsible both for reduced glutamate transport and the high permeability phenotype.

PCMBS was found to inhibit aspartate transport by system X^-_{AG} in Ed-A1 and CHO-K1. It does not follow that this compound need also inhibit the proposed water flux through system X^-_{AG}, as is the case for aquaporins, because a solute transporter would be expected to have a fundamentally different structure than that of an aquaporin. PCMBS has been reported to inhibit system L, a neutral amino acid transporter, in CHO-K1 (Campbell, Yu & Oxender, 1992), as well as a variety of amino acid transport systems of hepatocytes (Chiles, Dudeck-Collart & Kilberg, 1988). It is generally assumed that low affinity uptake of a substrate, modeled here by k_D, is mediated by other transport systems; for example, asparate may be a low affinity substrate of system L. Thus, the reduction in k_D by PCMBS suggests that several other transport systems were inhibited in these cells. It is, therefore, possible that covalent modification of multiple membrane activities by PCMBS is responsible for the general slow increase in osmotic sensitivities seen in both Ed-A1 and CHO-K1 by steric C-FFF. It is also possible that PCMBS inhibited systems responsible for producing Regulatory Volume Decreases and thereby exaggerated the swelling responses in both cell lines.

Steric FFF Analysis

The ability of steric C-FFF to detect cell swelling is noteworthy. While this technique cannot provide direct information on swelling rates as fast as those encountered here, it is clearly able to report reliably on differences in swelling produced, for instance, by mutation or sulfhydryl inhibitors. The photomicroscopic procedure used was limited to providing information on relatively few cells and it missed early time points. Steric C-FFF can report on thousdands of cells per run and many runs can be performed per day. Fluorescence-activated cell sorter (FACS) analysis of light scattering would be expected to detect the final volume changes encountered here, although relating the scatter signal to volume changes can require careful calibration (McGann, Walterson & Hogg, 1988). The design of standard FACS machines, however, is not well suited for monitoring rapid swelling. Detailed analysis of swelling of macrophages attached to coverslips was achieved by following changes in light scattering using an inverted microscope that allowed subsequent calibration of the scatter signal by confocal microscopy (Echevarria & Verkman, 1992). NMR (Waldeck et al., 1995) and ESR (Macey & Karan, 1993) procedures have been used to follow water fluxes in red cells with time resolution much less than a second. The application of such analytical methods to our cells would be desirable in the future to obtain detailed information on the mechanisms of water flow in the wild-type and mutant cells.

Steric C-FFF was able to separate a mixture of Ed-A1 and CHO-K1 cells when hypotonic elution buffers were used. However, unlike the case for polystyrene particles, baseline separations were not achieved for the cells. This unlikely has several causes, including the limited time period over which size differences persist and a wider natural variation in particle size. In spite of these constraints, separations sufficient for detecting differences in swelling were achieved and this technique should be adaptable to other situations. It is possible that exploration of other elution strategies, such as injecting a brief pulse of hypotonic saline in the carrier to catch fast swelling cells, could increase separation efficiences.

The problem of isolating a particular cell type is common in biological and biomedical research. Solutions have been sought in a wide variety of mechanical, physical, chemical, and biological approaches. Steric FFF was first applied in cell separation and characterization in 1984 by Caldwell et al. to separate red cells from different species (Caldwell et al., 1984). Since then, other (Andreux et al., 1993; Bigelow et al., 1989; Hoffstetter-Kuhn et al., 1992) applications have emerged, notably the 1 g form, which requires much simpler equipment (Andreux et al., 1993; Yue et al., 1994). In fact, preliminary experiments indicate that the swelling difference between Ed-A1 and CHO-K1 can be detected using 1 g steric FFF. Steric FFF has also been combined with other reagents and techniques, such as microspheres (*unpublished data*) and cellular adhesion chromatography (Bigelow et al., 1989), to achieve effective separations. The results reported here have demonstrated the first successful application of the steric FFF technique in a cell separation based on a transient physical property: differential swelling kinetics. Using the temporary size difference between CHO-K1 and Ed-A1 when exposed to a hypotonic environment, steric C-FFF rapidly sepa-

rated these two otherwise physically indistinguishable cell types and proved to be a useful analytical tool.

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