Optical Measurement of Osmotic Water Transport in Cultured Cells

Role of Glucose Transporters

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ABSTRACT Methodology was developed to measure osmotic water permeability in monolayer cultured cells and applied to examine the proposed role of glucose transporters in the water pathway (1989. *Proc. Natl. Acad. Sci. USA.* 86:8397–8401). J774 macrophages were grown on glass coverslips and mounted in a channel-type perfusion chamber for rapid fluid exchange without cell detachment. Relative cell volume was measured by 45° light scattering using an inverted microscope; measurement accuracy was validated by confocal imaging microscopy. The time required for >90% fluid exchange was <1 s. In response to a decrease in perfusate osmolality from 300 to 210 mosM, cells swelled without lag at an initial rate of 4.5%/s, corresponding to a water permeability coefficient of $(6.3 \pm 0.4) \times 10^{-3}$ cm/s (SE, $n = 20$, 23°C), assuming a cell surface-to-volume ratio of 4,400 cm^{-1}. The initial rate of cell swelling was proportional to osmotic gradient size, independent of perfusate viscosity, and increased by amphotericin B (25 μg/ml), and had an activation energy of 10.0 ± 1 kcal/mol (12–39°C). The compounds phloretin (20 μM) and cytochalasin B (2.5 μg/ml) inhibited glucose transport by >85% but did not influence P_f in paired experiments in which P_f was measured before and after inhibitor addition. The mercurials $HgCl_2$ (0.1 mM) and *p*-chloromercuribenzoate (1 mM) did not inhibit P_f. A stopped-flow light scattering technique was used to measure P_f independently in J774 macrophages grown in suspension culture. P_f in suspended cells was $(4.4 \pm 0.3) \times 10^{-3}$ cm/s (assuming a surface-to-volume ratio of 8,800 cm^{-1}), increased more than threefold by amphotericin B, and not inhibited by phloretin and cytochalasin B under conditions of strong inhibition of glucose transport. The glucose reflection coefficient was 0.98 ± 0.03 as measured by induced osmosis, assuming a unity reflection coefficient for sucrose. These results establish a quantitative method for measurement of osmotic water transport in adherent cultured cells and provide evidence that glucose transporters are not involved in the water transporting pathway.

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INTRODUCTION

A specialized pore or channel for plasma membrane water transport exists in the erythrocyte, kidney, and amphibian urinary bladder (for review, see Finkelstein, 1987; Verkman, 1989). Water permeability in these cells is high and strongly inhibited by mercurial sulfhydryl reagents. Further evidence supporting the existence of a water-filled conduit is a ratio of osmotic-to-diffusional water permeability (P_f/P_d) > 1 and a low activation energy (E_a < 6 kcal/mol). Recent studies of water channel expression in *Xenopus* oocytes injected with mRNA from reticulocyte, kidney, and toad bladder suggest that the water channel is a protein (Zhang, Logee, and Verkman, 1990; Zhang and Verkman, 1991). Specialized water transporting pathways in kidney and amphibian urinary bladder are essential for rapid and regulated transcellular volume flow (Handler, 1988; Verkman, Lencer, Brown, and Ausiello, 1988; Shi and Verkman, 1989; Shi, Wang, and Verkman, 1990); rapid water transport in the erythrocyte has been proposed to be important for osmotic volume equilibration as erythrocytes pass through the hypertonic renal medulla (Macey, 1984).

The existence of specialized or dedicated water transporting pathways in other cell types has been less certain. Because water transport in artificial bilayers (P_f = 5–50 × 10^{-4} cm/s) is not much lower than that in membranes containing mercurial-sensitive water channels (100–300 × 10^{-4} cm/s) (Finkelstein, 1987), there is no compelling reason to predict that most cells would contain water channels. Most cells do not have rapid vectorial water transport, are not exposed to rapidly changing osmotic gradients, and require minimal water permeability (P_f < 10^{-4} cm/s) for volume regulation. Fischbarg and co-workers (Fischbarg, Liebovilch, and Koniarek, 1987; Fischbarg, Kunyan, Hirsch, Lecuona, Rogozinski, Silverstein, and Loike, 1989; Fischbarg, Kunyan, Vera, Arant, Silverstein, Loike, and Rosen, 1990) proposed that the ubiquitous Na-independent glucose transporter may be a universal water channel in many cell types. Their principle evidence was that: (*a*) the potent glucose transport inhibitors phloretin and cytochalasin B inhibited water transport in cultured J774 macrophages and rabbit corneal endothelium, and (*b*) expression in *Xenopus* oocytes of mRNA in vitro transcribed from cDNA encoding glucose transporters caused a small (~25%) increase in oocyte osmotic water permeability. If correct, the hypothesis that water passes through glucose transporters would have general biological significance for regulatory processes in many cell types.

We report here the development and validation of methodology to measure osmotic water permeability in adherent cultured cells. The method is an extension of a laser light scattering method introduced by Fischbarg et al. (1989). The measurement of water permeability in cultured cells is important for the development of cultured cells lines that retain in vivo water transport characteristics and are suitable for transfection with mammalian expression vectors containing water channel cDNA. The methodology was used to reexamine whether cytochalasin B, a potent and selective inhibitor of glucose transport, and phloretin, a potent though nonselective inhibitor, inhibit water transport in cultured adherent J774 macrophages. Under conditions of nearly complete inhibition of glucose transport, there was no inhibition of osmotic water transport. In addition, macrophage water permeability was not inhibited by mercurials and had an activation energy consistent with a lipid diffusion

pathway. Similar results were obtained in stopped-flow light scattering measurements of J774 macrophages grown in suspension culture. In separate oocyte expression studies (Zhang, Alper, Thorens, and Verkman, 1991), we confirmed that overexpression of glucose transporters increased water permeability, but concluded that the contribution of glucose transporters to total water permeability in cell membranes is very small (see Discussion).

METHODS

Cell Culture

J774 macrophages (No. TIB-67; American Type Culture Collection, Rockville, MD) were grown on 18-mm-diam round glass coverslips or plastic dishes in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum. Cells were grown in a 5% CO_2 incubator at 37°C and used when nearly confluent (5–7 d after plating, cell density ~3,600 cells/mm^2). For experiments on suspended cells, J774 macrophages in 200 ml of media were grown at 37°C in 2-liter sealed roller bottles that were rotated continuously at 0.3

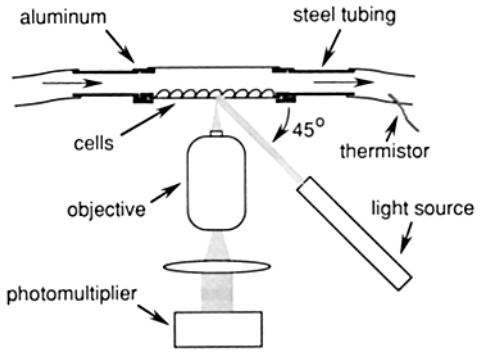

FIGURE 1. Schematic of light scattering apparatus. The perfusion chamber was a 2-mm rectangular channel to give laminar fluid flow. Cells were illuminated at a 45° incident angle; light scattering was detected by an inverted microscope. See text for details.

rotations/min. Cells were used when the density was 2×10^6/ml, at which time >95% of the cells excluded the vital stain Trypan blue.

Light Scattering Microscopy Measurements

Coverslips containing cultured cells were mounted in an aluminum perfusion chamber in which the perfusion path consisted of a 2-mm-wide rectangular channel to give laminar fluid flow (Fig. 1). The coverslip containing cells facing upward made up the bottom surface of the chamber. The chamber was perfused continuously at 7 ml/min by a gravity-driven system. The time required for 50% solution exchange was <0.4 s (see Results). Perfusate temperature was monitored by a small thermistor at the distal end of the chamber.

The perfusion chamber was placed on the stage of a Nikon inverted microscope so that the cell-free surface of the glass coverslip was nearest the objective. Cells were illuminated from below at an angle of 45° from the plane of the cell monolayer. The illumination source consisted of a 50-W tungsten–halogen lamp powered by a stabilized direct current power supply (intensity variation < 0.01%) (Oriel Corp., Stratford, CT) and focused onto the cells by a convex lens with 20 cm focal length. The excitation light was filtered to give wavelengths of 640–700 nm. In some experiments the excitation source consisted of a He-Ne laser (1 mW at

642 nm). Cells were viewed with a ×25 long working distance objective modified for bright-field Hoffman optics (N.A. 0.35; E. Leitz, Inc., Rockleigh, NJ). The intensity of scattered light was detected at a maximum rate of 30 points/s by a photomultiplier as described previously (Kuwahara, Berry, and Verkman, 1988).

The composition of the isosmotic perfusion buffer (buffer A) was (mM): 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1.5 $MgSO_4$, 1 KH_2PO_4, and 5 glucose, titrated to pH 7.4 with NaOH. Hypoosmotic solutions were obtained by dilution of the isosmotic buffer with water. Some solutions also contained the compounds phloretin, cytochalasin B, amphotericin B, ouabain, $HgCl_2$, or pCMB which were prepared just before the experiment.

In some experiments, cell images were detected by a silicon intensified target camera (SIT-66; DAGE-MTI, Michigan City, IN) and processed by image analysis hardware and software from Data Translation Inc. (Marlboro, MA). Cells were loaded with 2,7-bis-carboxyethyl-5[and 6]-carboxyfluorescein (BCECF) by a 10-min incubation with 2 μM BCECF-acetoxymethylester at 37°C and viewed with a Nipkow wheel confocal microscope (Technical Instruments Co., San Francisco, CA) through a ×60 oil immersion objective (N.A. 1.4; Nikon Inc., Garden City, NY). Relative cell volume was estimated from measured relative area by a geometric model.[1]

The osmotic water permeability coefficient (P_f) was determined from the relation,

$$P_f = \frac{1}{(S/V_o)V_w(C_{in} - C_{out})} \frac{d(V/V_o)}{dt} \quad (1)$$

where S/V_o is cell surface-to-volume ratio estimated to be 4,400 cm^{-1} (Fischbarg et al., 1989; also see Results), V_w is the partial molar volume of water (18 cm^3/mol), $(C_{in}-C_{out})$ is the initial osmotic gradient, and $d(V/V_o)/dt$ is the initial rate of relative cell volume change determined from the initial slope of light scattering vs. time data after addition of an osmotic gradient, and the calibration between scattered light intensity and cell volume (see Results). The initial slope of the light scattering vs. time data was estimated from the initial slope of a quadratic polynomial fitted to scattering intensities measured during the first 5 s after the solution exchange. The nonosmotic volume of J774 cells in suspension was estimated by measurement of cell volume by an Elzone Particle Counter (Particle Data, Inc., Elmhurst, IL) at 1 min after addition of external sucrose to give external osmolalities of 300–1,000 mosM. Extrapolation of a linear volume vs. inverse osmolality plot as described by Meyer and Verkman (1987) gave a nonosmotic volume equal to 14% of isotonic volume. In Eq. 1, V_o represents the osmotically active cell volume, which is 86% of apparent isotonic volume.

Stopped-Flow Transport Measurements

Osmotic water permeability in suspended J774 macrophages was measured by a stopped-flow light scattering technique using a stopped-flow apparatus (model SF-51; Hi-Tech, Wiltshire, UK). 0.075 ml of a suspension of J774 macrophages (1–2 × 10^7/ml) in buffer A was mixed in < 1 ms with an equal volume of buffer A containing 200 mM sucrose to give a 100-mM inwardly directed sucrose gradient. The time course of 90° scattered light intensity at 520 nm was recorded by a MINC/23 computer. The instrument dead time was 1.3 ms and the maximum rate of data acquisition was 0.1 ms/point. Generally, 10 serial experiments were averaged for

[1] As a simple geometric approximation, assume the cell is always a hemisphere. Initially the hemisphere radius is r_0, cell volume is $\frac{2}{3}\pi r_0^3$, and the area in the focal plane at a distance $r_0/2$ above the cell base is $\frac{3}{4}\pi r_0^2$. When the cell swells to a radius r, cell volume is $\frac{2}{3}\pi r^2$ and the area in the fixed focal plane is $\pi(r^2 - r_0^2/4)$. The relative area in the focal plane, A_{rel}, is thus $\frac{4}{3}(r/r_0)^2 - \frac{1}{3}$ after swelling. Rearranging, the relative volume V/V_0 $(= r^3/r_0^3)$ is equal to $(3A_{rel} - 1)^{3/2}/8$.

each measurement. Temperature was controlled by a circulating water bath and measured by an indwelling thermistor. P_f was calculated from the time course of scattered light intensity and a surface-to-volume ratio of 8,800 cm^{-1} (assumed to be twice that of the adherent cells) by a fitting procedure reported in detail previously (Meyer and Verkman, 1987). If a J774 macrophage were perfectly smooth, the measured diameter of 12 μm would predict a surface-to-volume ratio of 5,000 cm^{-1}. The calculation of P_f assumes a homogeneous cell population with respect to size and water transporting properties, and a linear relationship between cell volume and scattered light intensity. The latter assumption was validated in J774 macrophages by demonstrating a linear decrease in the amplitude of the light scattering curve with the reciprocal osmolality (proportional to final cell volume) of solutions (in the range 300–400 mosM) after mixture in the stopped-flow apparatus.

The glucose reflection coefficient was measured by the induced osmosis "null point" method of Pearce and Verkman (1989) which is not sensitive to refractive index artifacts. J774 macrophages suspended in buffer A were mixed rapidly with an equal volume of sucrose or D-glucose in distilled water (200–400 mM). The initial slopes of the light scattering vs. time curves were measured. The glucose reflection coefficient was determined from the ratio of interpolated concentrations of sucrose to glucose required to give zero initial slope. It was assumed that sucrose had a unity reflection coefficient.

Glucose Uptake Measurements

Uptake of 3-*O*-[methyl-^{3}H]-D-glucose (Me-Glu) (sp act 79 Ci/mmol; New England Nuclear, Boston, MA) was studied in J774 macrophages grown on plastic six-well dishes or in suspension. When present, inhibitors were added 15 min before Me-Glu addition. To initiate influx in the adherent cells, 1 μCi Me-Glu was added to 1 ml of buffer A (containing 5 mM glucose) and the dish was rocked continuously at 23°C. After specified incubation times the cells were washed four times with 3 ml of ice-cold buffer A containing 20 μM cytochalasin B (total washing time < 30 s) and cells were disrupted by addition of 0.1 N NaOH. A 0.1-ml sample was dissolved in scintillation fluid for determination of ^{3}H radioactivity. To initiate influx in the suspended cells, 5 μCi of Me-Glu was added to 1 ml of cell suspension in buffer A ($2–4 \times 10^7$ cells/ml). 100-μl aliquots were removed at specified times, vacuum filtered through 1-μm filters (Millipore Corp., Bedford, MA), and washed three times with 2 ml of cold buffer A containing cytochalasin B (washing time < 10 s). The filters were dissolved in scintillation fluid for determination of radioactivity. Counts were corrected for nonspecific Me-Glu binding (<5%).

RESULTS

The first set of experiments was performed to show that the intensity of light scattered from a monolayer of J774 macrophages provided a quantitative measure of cell volume. A glass coverslip containing the cells was mounted as shown in Fig. 1 and illuminated at an incident angle of 45°. Fig. 2 (*top*) shows photomicrographs of a group of cells viewed by Hoffman bright-field optics (*left*) and 45° light scattering (*right*). The cells appeared dome-shaped with fairly uniform diameter. Light was scattered strongly from individual cells with little background signal from the coverglass.

In response to an osmotic gradient to induce cell swelling (Fig. 3 *A*), there was a time-dependent decrease in scattered light intensity with signal-to-noise ratio > 100. In general, the intensity of scattered light depends on cell geometry and solution refractive index. The influence of refractive index was examined by addition of 3% dextran to the perfusate (Fig. 3 *B*). The change in refractive index caused by dextran

addition (1.3337 to 1.3382) was fivefold greater than that caused by dilution of the isosmotic buffer (1.3337 to 1.3328). Dextran caused a rapid and small decrease in light scattering, indicating that the main determinant of light scattering was cell geometry.

To show that the time course of light scattering in Fig. 3 *A* corresponded to cell swelling, independent measurements of cell volume were obtained by confocal fluorescence microscopy. Fig. 2 (*bottom*) shows confocal micrographs of cells whose interior volume was stained with the fluorescent indicator BCECF. Images were

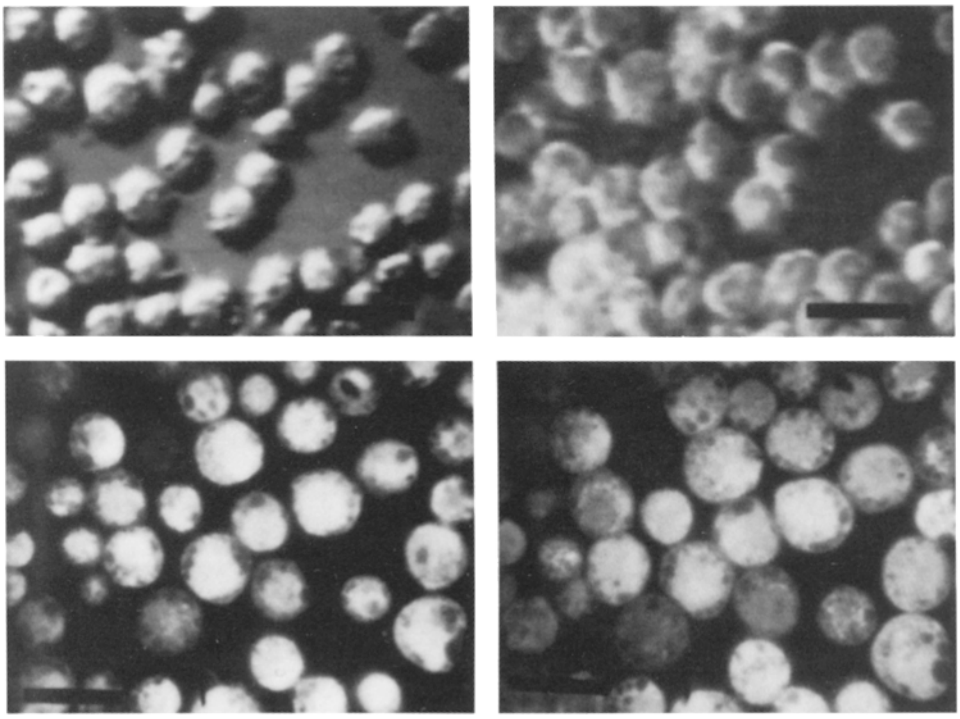

FIGURE 2. Micrographs of adherent J774 macrophages. (*Top*) Micrographs obtained by bright-field Hoffman optics (*left*) and 45° light scattering (*right*), ×25 objective. (*Bottom*) Confocal epifluorescence micrographs of cells loaded with BCECF in isosmotic buffer (300 mosM, *left*) and hypoosmotic buffer (210 mosM, *right*), ×60 objective. Scale bar: 10 μm.

obtained at normal cell volume (300 mosM buffer, *left*) and 45 s after swelling in 210 mosM buffer (*right*). The plane of the optical section was approximately half-way between the cell base and apex. Relative cell volume was estimated from the relative cell area measured by quantitative image analysis as described in footnote 1.

Fig. 3 *C* shows a comparison of the time course of relative cell volume determined from a series of timed confocal measurements made on 20 individual cells and from the area-integrated light scattering data in Fig. 3 *A*. The light scattering data were converted to relative cell volume by a calibration procedure described below, taking into account the presence of nonosmotic cell volume (see Methods). The close

correspondence between cell volumes determined by light scattering and confocal microscopy supports the conclusion that the major determinant of light scattering is cell volume and that measurement of light scattering provides a continuous and high resolution record of cell volume. Further experiments were carried out to show that the time course of scattered light intensity in response to osmotic gradients can be interpreted in terms of plasma membrane osmotic water permeability.

To measure osmotic water permeability quantitatively, the time required to establish an osmotic gradient (solution exchange time) must be much less than the subsequent time course of cell volume change. Fig. 4 *A* shows that the time for 50% solution exchange, as measured by the time course of absorbance upon switching

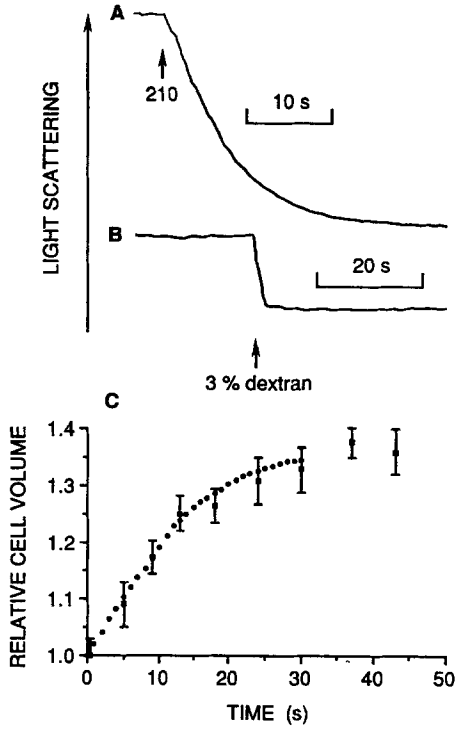

FIGURE 3. Time course of cell volume in response to a decrease in perfusate osmolality from 300 to 210 mosM. (*A*) 45° light scattering. Solutions were perfused continuously at 7–10 ml/min and switched at the arrow (see Methods). The signal decreased by 6.8%. (*B*) Effect of addition of 3% dextran (M_r 48,000) to the isosmotic buffer without change in osmolality. The scale on the ordinate is the same as in *A*. (*C*) Comparison of cell swelling time course measured by light scattering and confocal fluorescence microscopy (mean ± SE). Light scattering data from four experiments were converted to relative cell volume by use of the calibration relation in Fig. 5 *B* and the measured nonosmotic volume equal to 14% of isosmotic volume. Confocal microscopy data from two experiments (data from 20 separate cells averaged) were converted to relative cell volume by the geometric model given in footnote 1.

between solutions containing water or 10 μM rhodamine B, was ~0.4 s at the perfusion rate used in cell studies. The 90% exchange time (~1 s) was much faster than the half-time for cell swelling or shrinking of ~10 s (Fig. 4 *B*). Fig. 4 *B* also shows absence of a significant lag time between establishing the osmotic gradient and maximal water flow, suggesting absence of an extracellular unstirred layer that would restrict water permeability (Barry and Diamond, 1984). As described in Methods, quantitative determination of water permeability required the measurement of the light scattering vs. time slope immediately after the solution exchange.

In the absence of unstirred layer effects, nonequilibrium thermodynamics predicts a linear relationship between osmotic gradient size and induced volume flow (Kedem

and Katchalsky, 1958). Fig. 4 *C* shows the cell response to osmotic challenges of 60, 90, and 180 mosM (cell > perfusate osmolality). Unless otherwise indicated, experiments were performed at 23°C. The initial rate of decrease in scattered light intensity, proportional to volume flow, was linearly related to osmotic gradient size

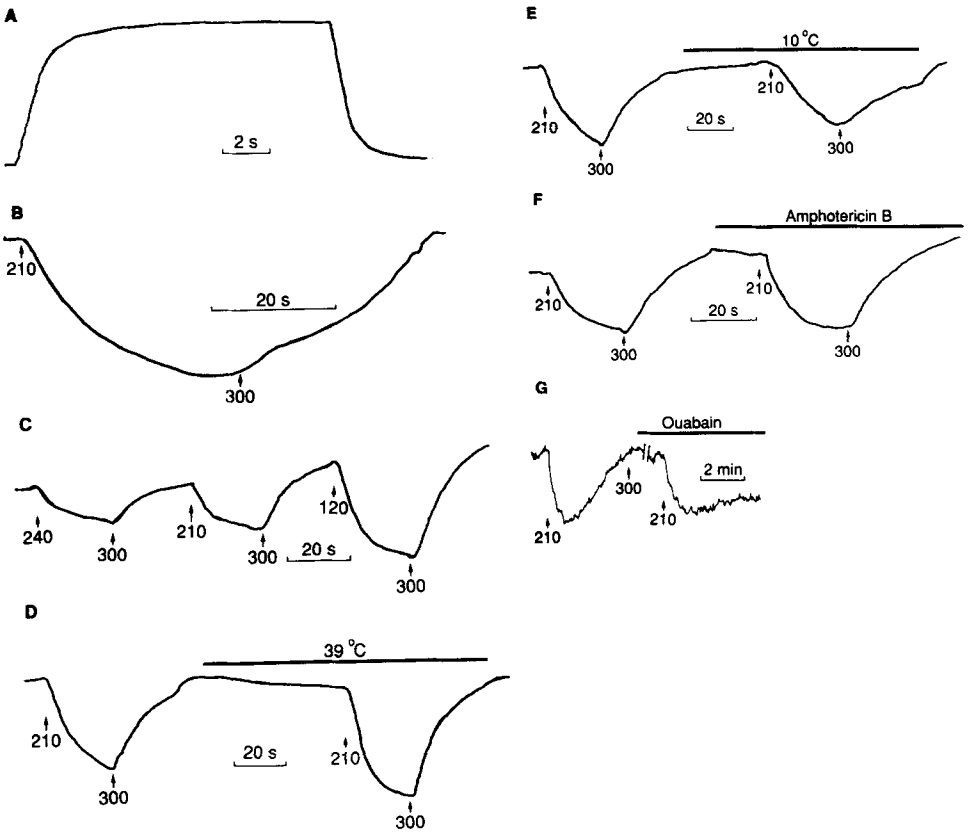

FIGURE 4. Osmotic water permeability of adherent J774 macrophages measured by light scattering. Experiments were performed at 23°C unless otherwise specified. (*A*) Solution exchange time determined by measurement of transmitted light (580 nm) upon removal and addition of 10 μM rhodamine B to the perfusate. (*B*) Reversible swelling and shrinking of J774 macrophages measured by 45° light scattering. (*C*) Swelling and shrinking in response to osmotic gradients of different magnitude. (*D* and *E*) Effect of temperature on the time course of cell swelling and shrinking. (*F*) Effect of amphotericin B (25 μg/ml) on cell swelling and shrinking. (*G*) Spontaneous volume regulation shown in the absence but not in the presence of 1 mM ouabain.

(Fig. 5 *A*). The amplitudes of the decrease in relative light scattering for gradients of different size provided an empirical calibration for conversion of scattering intensities to cell volume (Fig. 5 *B*). Relative light scattering was related to relative osmotic cell volume by a curvilinear calibration, especially for large perturbations from the isosmotic volume. The data in Fig. 5, *A* and *B*, taken together with the cell

surface-to-volume ratio (see Methods), gave a P_f value of (6.3 ± 0.4) × 10^{-3} cm/s (SE, 20 experiments) at 23°C. This P_f value should be regarded as an upper limit to the actual P_f because there may be plasma membrane invaginations, which would increase cell surface-to-volume ratio, that were not taken into account. Addition of 4% dextran (M_r 64,000) to perfusion solutions to increase viscosity approximately threefold without changing osmolality did not alter P_f significantly, (6.0 ± 0.6) × 10^{-3}

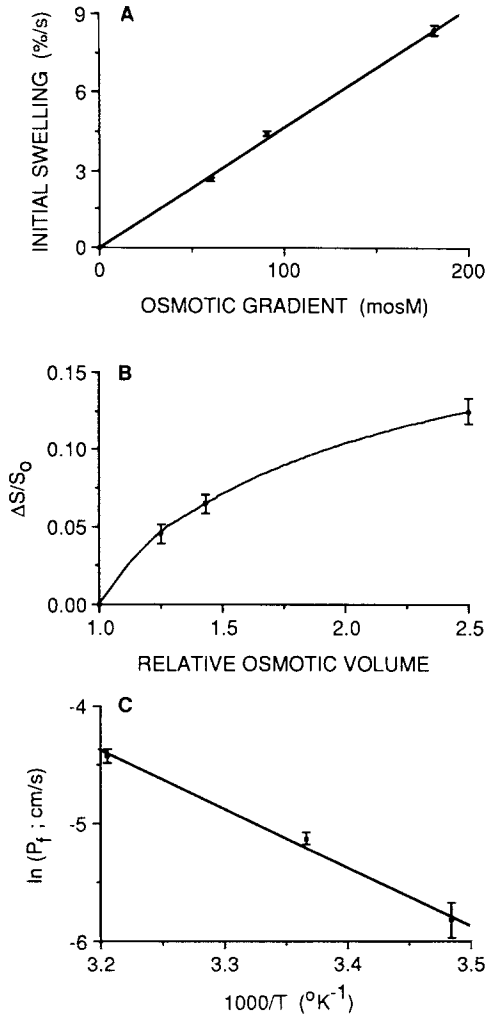

FIGURE 5. Characteristics of water permeability in J774 macrophages. (*A*) Dependence of the initial rate of osmotic water efflux on osmotic gradient size. The ordinate is the relative initial slope of the light scattering time course as in Fig. 4 *C*. Data are mean ± SE for four sets of measurements performed on different coverslips shown with fitted line. (*B*) Dependence of relative signal amplitude (mean ± SE, four measurements) on relative osmotic cell volume, calculated from the ratio of perfusate osmolality to initial cell osmolality (300 mosM). Data were fitted to a cubic spline. (*C*) Arrhenius plot for the temperature dependence of P_f. Each point is the mean ± SE for three to five sets of measurements; the fitted activation energy was 10.0 ± 1 kcal/mol.

cm/s (n = 3), providing further support for the conclusion that unstirred layers are absent.

The activation energy (E_a) for P_f has been used to distinguish between membrane water transporting pathways mediated by a pore or channel, and a lipid-diffusion mechanism (Finkelstein, 1987). Fig. 4, *D* and *E*, shows significant effects of temperature on water influx and efflux rates; the data from five sets of measurements are

summarized in an Arrhenius plot (Fig. 5 *C*). The data give a single E_a of 10.0 ± 1 kcal/mol in the temperature range 10–39°C. This value is much higher than the E_a predicted for water passage through a pore or channel (2–5 kcal/mol, Verkman, 1989; Finkelstein, 1987) or if water transport were limited by an unstirred layer which caused solute polarization (~5 kcal/mol) (Barry and Diamond, 1984).

Additional evidence that the cell plasma membrane is the rate-limiting barrier to water flow was obtained by addition of a low concentration of the pore-forming agent amphotericin B (Finkelstein, 1974) to the perfusate (Fig. 4 *F*). In four sets of measurements, 25 μg/ml amphotericin B increased P_f to (130 ± 15) × 10^{-4} cm/s (23°C). In separate control studies in which perfusate osmolality was repeatedly switched between 210 and 300 mosM every 30 s, there was no systematic change in P_f measured for >30 min. However, a prolonged incubation in hypoosmotic perfusate was associated with a spontaneous decrease in cell volume over ~3 min due to activation of volume regulatory mechanisms (Fig. 4 *G*). Volume regulation was abolished by addition of ouabain to inhibit the Na/K ATPase.

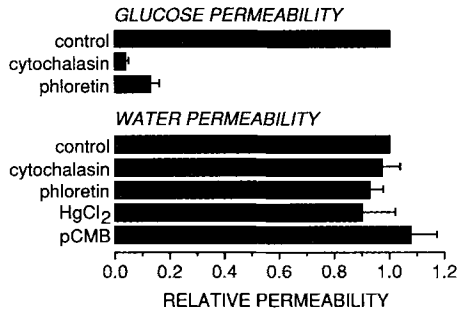

FIGURE 6. Inhibitor effects on glucose and osmotic water permeability in adherent J774 macrophages. Data (mean ± SE for three separate sets of experiments) are shown for paired studies in which control (no inhibitor) was assigned a relative permeability of 1. Methylglucose transport was measured by 5-min uptake. Osmotic water permeability was measured by 45° light scattering. Inhibitor concentrations were: 2.5 μg/ml cytochalasin B, 20 μM phloretin, 0.1 mM $HgCl_2$, and 1 mM pCMB.

The next set of studies was performed to examine the influence of glucose transport inhibitors on water transport. Uptake of radiolabeled methylglucose in the J774 cell monolayers was linear for 5–10 min and 50% complete in ~12 min, corresponding to a methylglucose permeability coefficient of ~3 × 10^{-7} cm/s at 23°C. Uptake of methylglucose at 5 min was strongly inhibited by cytochalasin B (2.5 μg/ml) and phloretin (20 μM) (Fig. 6), indicating the presence of a Na-independent glucose transporter as reported by Fischbarg et al. (1989). The data in Fig. 6 are expressed as the permeability in the presence of inhibitor relative to that of control cells without inhibitor (relative permeability 1.0).

Water transport was measured by a paired protocol in which P_f was measured in the same group of cells before and after inhibitor addition (Fig. 7). Under conditions of strong inhibition of glucose transport, cytochalasin B and phloretin did not inhibit water transport significantly (Fig. 7, *A* and *B*). The mercurial inhibitors of water transport in erythrocytes and kidney cells, $HgCl_2$ and pCMB (Macey, 1984; Verkman, 1989), also did not inhibit macrophage water transport. Water permeabilities for a series of paired studies are summarized in Fig. 6, in which control cells (before inhibitor addition) were assigned a relative permeability of 1.

A set of independent water transport and methylglucose uptake experiments were carried out in J774 cells in suspension that were grown in roller bottles. Fig. 8 shows a stopped-flow light scattering study in which cells were subjected to a 100-mosM inwardly directed sucrose gradient. The osmotic gradient caused osmotic water efflux and an increase in scattered light intensity; cell volume decreased by ~25% at equilibrium. There was no change in scattered light intensity in the absence of an

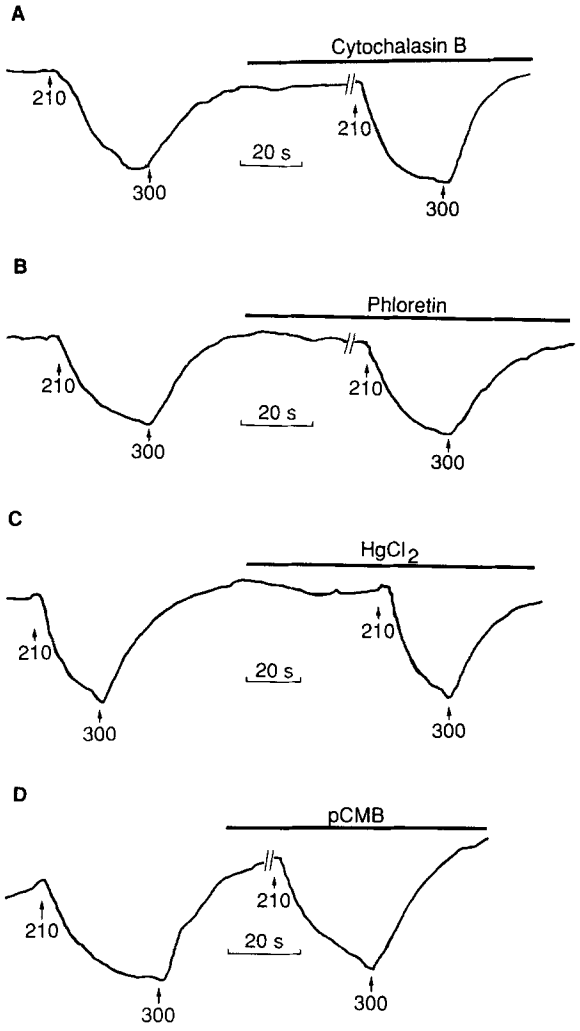

FIGURE 7. Influence of glucose transport inhibitors and mercurials on osmotic water transport in J774 macrophages. Inhibitor concentrations were as given in the legend to Fig. 6. The time between inhibitor addition and water transport measurements was 15 min (cytochalasin B, phloretin, and pCMB; note break in curves) and 1 min ($HgCl_2$).

osmotic gradient (not shown). Preincubation of cells with cytochalasin B or phloretin did not effect the time course of light scattering significantly. In three separate sets of measurements (10 experiments averaged in each set) performed in J774 macrophage suspensions prepared from different cell cultures, P_f (in cm/s × 10^{-3}, mean ± SE, n = 3) at 23°C was: 4.4 ± 0.3 (control), 4.2 ± 0.3 (2.5 μg/ml cytochalasin B), and 4.3 ± 0.2 (20 μM phloretin). Uptake of radiolabeled methylglucose was 50%

complete in 5–6 min. Compared with control (100%), 3-min uptake of methylglucose was 16 ± 4% (cytochalasin B) and 26 ± 7% (phloretin). In the absence of inhibitors, uptake of methylglucose was 22 ± 4% of control in the presence of 25 mM glucose, indicating that glucose and methylglucose share the same carrier.

Fig. 8 shows that preincubation of cells with amphotericin B increased P_f approximately threefold, indicating that unstirred layer effects on water permeability were minimal or absent. Similarly, increasing external viscosity approximately threefold by addition of dextran (M_r 64,000) did not affect P_f (not shown). In the absence of amphotericin B, P_f was strongly temperature dependent, decreasing to (2.6 ± 0.2) × 10^{-3} cm/s at 15°C. The reflection coefficient for glucose was measured by the induced osmosis method (Pearce and Verkman, 1989) from the relative concentrations of sucrose and glucose needed to give zero initial volume change (null point, see

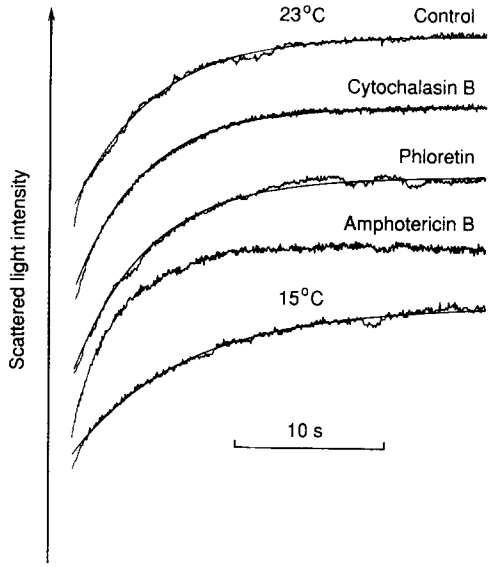

FIGURE 8. Osmotic water permeability in suspended J774 macrophages measured by a stopped-flow light scattering technique (see Methods). Cells were subjected to a 100-mM inwardly directed gradient of sucrose at 23°C (top four curves) or at 15°C (bottom curve). Where indicated, cytochalasin B (2.5 μg/ml), phloretin (20 μM), or amphotericin B (50 μg/ml) were added 10 min before stopped-flow measurements. Each curve is the average of 10 experiments performed on one set of J774 suspension cultures, typical of three. Mean data for all three sets of cultures are given in the text.

Methods). In three sets of experiments, the glucose reflection coefficient was 0.98 ± 0.03.

DISCUSSION

The initial goal of our study was to validate methodology for measurement of osmotic water permeability in cultured cells grown on a glass coverslip. Measurement of osmotic water permeability requires the determination of the time course of cell volume in response to osmotic gradients. In intact epithelia including kidney tubules and gallbladder, cell volume measurements have been performed by tracing cell boundaries in images obtained by differential interference contrast microscopy (Strange and Spring, 1987). This technique is relatively laborious and cannot be easily applied to adherent monolayer cells because of fixed attachment points at the base of cells. Bright-field confocal microscopy can in principle be used to define cell boundaries at a series of z-planes for three-dimensional image reconstruction (Agard,

Hiraoka, Shaw, and Sedat, 1989), however boundary contrast is very low and real-time imaging of rapid volume changes is not practical. Based on the work of Fischbarg et al. (1989), we chose to apply a light scattering technique to obtain a continuous record of cell volume. Light scattering methods have been used extensively in measurements of water permeability in suspended cells and sealed membrane vesicles using rapid mixing devices (Mlekoday, Moore, and Levitt, 1983; Verkman, 1989). With suitable calibration, the time course of light scattering provides quantitative information about plasma membrane water permeability without the necessity of processing large image data arrays.

The intensity of light scattered from an adherent cell depends in a complex manner upon cell geometry, solution and membrane refractive index, intracellular structures, wavelength, and optical configuration. Cell geometry, membrane refractive index, and the characteristics of intracellular structures are not subject to experimental manipulation. The wavelength of incident light and the optical configuration were optimized to maximize the dependence of scattered light intensity on cell volume. Low intensity light of long wavelength minimized photodynamic cell injury. The 45° angle between the incident beam and detector was convenient for measurement of scattered light intensity by an inverted microscope; a microscope was chosen for efficient collection of light by objective lenses and for preservation of spatial information in imaging applications. For cell illumination, a tungsten–halogen light source rather than a laser was chosen because of its improved stability and uniform (nonspecular) beam profile. Solution refractive index in the range used here was found to be of minor importance because addition of dextran (to change refractive index without altering cell volume) had little effect on scattered light intensity.

Important design criteria for the perfusion chamber were rapid fluid exchange without extracellular unstirred layers or traumatic cell detachment. A single-channel, laminar flow chamber was constructed based on the design of Strange and Spring (1986) reported for perfusion of kidney tubules. At a 7–10 ml/min perfusion rate, there was no cell detachment and the half-time for solution exchange was <0.5 s. Several lines of evidence support the conclusion that unstirred layer effects were minimal (Barry and Diamond, 1984): (*a*) there was no lag in the light scattering response after a change in perfusate osmolality, (*b*) E_a was 10 kcal/mol, much higher than that predicted if P_f were limited by an unstirred layer (~ 5 kcal/mol), (*c*) volume flow was linearly related to osmotic gradient size, (*d*) P_f increased with insertion of amphotericin B water channels, and (*e*) P_f was not altered by a threefold increase in perfusate viscosity. Therefore the measured P_f is an intrinsic property of the plasma membrane rather than of a serial resistance or a rate-limiting mixing time or electronic response.

The second goal of our study was to evaluate whether water moved through the glucose pathway in cultured J774 macrophages, the cell line used by Fischbarg et al. (1989). Cells were grown in a manner similar to that described by Fischbarg et al. and demonstrated strong inhibition of glucose transport by low concentrations of phloretin and cytochalasin B. Experiments were performed in a paired manner in which the same cells were used for control and inhibitor studies. There was no significant effect of the glucose transport inhibitors on water permeability in the adherent J774

macrophages. In separate stopped-flow experiments performed on suspended J774 macrophages, there was no effect of glucose transport inhibitors on water permeability, and the glucose reflection coefficient was near unity. Our data therefore do not support the hypothesis that glucose transporters are water channels and that glucose and water share a common pathway.

It is more difficult to determine whether other channel-like pathways for water exist in the plasma membrane of J774 macrophages. The absolute P_f value is consistent with the lack of water channels, however the P_f values might be inaccurate by a factor of two or more because of uncertainties in the effective surface-to-volume ratios of the adherent and suspended macrophages. The P_f of J774 macrophages reported here (0.004–0.006 cm/s) is greater than that for lipid bilayers (P_f 0.0006 cm/s [cholesterol-PC] to 0.002 cm/s [PC]; Finkelstein, 1987), but comparable to P_f for plasma membranes from platelets, intestinal cells, and tracheal cells which do not contain water channels (P_f 0.003–0.005 cm/s; Verkman, 1989). The 0.004–0.006 cm/s value is lower than P_f for erythrocytes (P_f 0.02–0.05 cm/s; Macey, 1984; Tsai, Zhang, and Verkman, 1991) and membranes containing the vasopressin-sensitive water channel (P_f 0.04–0.1 cm/s; Verkman et al., 1988; Shi and Verkman, 1989). Macrophage P_f was not inhibited by mercurials. The activation energy (E_a) of ~ 10 kcal/mol measured here does not prove or disprove a channel-like pathway for water movement. An E_a of 2–5 kcal/mol has been measured in erythrocytes and kidney, where mercurial-sensitive water transporters exist (Verkman, 1989, 1992); there is a wide range of E_a (10–20 kcal/mol) values for membranes not containing water channels. Thus, it is concluded that the macrophage does not have an erythrocyte or kidney-like water channel; however, the presence of other novel proteins with water transporting properties cannot be excluded.

It is important to evaluate whether methodological differences could account for the differences between the results reported here and those published by Fischbarg et al. (1989). Both sets of cultured cells had high rates of glucose transport that were strongly inhibited by phloretin and cytochalasin B. Fischbarg et al. used a He-Ne laser for cell illumination and a 90° light scattering configuration. We repeated the experiments in Fig. 7 using a He-Ne laser and found no effects of phloretin and cytochalasin B on water transport. Another possible difference in the protocols was temperature. Most of our experiments were carried out at 23°C, whereas the studies Fischbarg et al. (1989) were performed at 37°C. The other experimental differences are slight differences in the composition of the hypotonic buffers, and the use here of a paired experimental protocol and a laminar flow perfusion chamber. The perfusion chamber may be an important difference. Our chamber had a volume of 0.1 ml and a half-time for fluid exchange of < 0.5 s. The chamber described by Fischbarg et al. (1989) had a 2-ml volume and a half-time for mixing of ~ 10 s, similar to the half-time for the change in scattered light intensity (10–15 s) in response to an osmotic gradient. Therefore, as suggested by the lag phase in their light scattering curves, the water permeabilities measured by Fischbarg et al. may have been influenced by unstirred layer effects.

Another methodological difference in the studies was the calculation of water permeability from the light scattering data. Fischbarg et al. (1989) fitted the complete light scattering vs. time curve, making use of a model that incorporated P_f, an

unstirred layer thickness, and a NaCl diffusion coefficient in the unstirred layer. Because of the absence of a lag phase in the experiments reported here, we determined P_f directly from the initial light scattering vs. time slope after a change in solution osmolality. The initial slope could be determined reproducibly in a series of measurements performed on independent cell cultures. We chose not to use an apparent half-time, or to fit the complete light scattering time course because the initial portion of the curve contained information about changing cell volume before significant collapse of the osmotic gradient, possible solute entry, and nonlinear changes in cell volume. For analysis of the stopped-flow data, the full light scattering vs. time curve was fitted according to a well-established set of equations (Macey, 1984; Meyer and Verkman, 1987). As shown in Fig. 8, the theoretical curve fitted the experimental data closely, except over the first few milliseconds, where the experimental curve increased faster than predicted by theory. As observed in other cell systems, this small increase is probably due to a small population of leaky nonviable cells, or small membrane fragments with high surface-to-volume ratios. Because of this uncertainty at early times, we calculated P_f by fitting the complete light scattering curve. Calculated P_f was reproducible in experiments performed on separate suspension cultures, and sensitive to temperature and addition of amphotericin B. In agreement with the adherent cell results, phloretin and cytochalasin B did not affect water permeability rates in suspended cells measured by stopped-flow light scattering.

Fischbarg et al. (1990) also reported that expression of in vitro transcribed mRNA encoding Na-independent glucose transporters in *Xenopus* oocytes caused a small increase in osmotic water permeability. As part of a study of the roles of band 3 and band 4.5 in erythrocyte water permeability, we confirmed that expression of glucose transporters increased oocyte water permeability (Zhang et al., 1991). When assayed at 10°C after a 72-h incubation, P_f of *Xenopus* oocytes microinjected with 50 ng of mRNA in vitro transcribed from cDNA encoding brain (GLUT1) or liver (GLUT2) glucose transporters increased from 4×10^{-4} cm/s to 13×10^{-4} cm/s. There was a >20-fold increase in oocyte glucose transport. Both water and glucose transport were strongly inhibited by phloretin and cytochalasin B. These results support the conclusion that the glucose transporter is associated with a small water conductance. In contrast, microinjection of unfractionated mRNA from reticulocyte or kidney, expected to contain <0.1% mRNA encoding water channels, gave a more than fourfold increase in oocyte P_f that was inhibited by mercurials but not by phloretin or cytochalasin B. In addition, suppression of GLUT1 expression by a 21-mer GLUT1 anti-sense deoxyribonucleotide did not affect the increase in oocyte P_f due to expression of reticulocyte mRNA, suggesting that the glucose transporter was not required for water channel function.

The osmotic water permeability in J774 macrophages that is due to glucose transporters can be estimated from the oocyte data because unstirred layer effects are absent in the oocyte water transport measurements (Zhang and Verkman, 1991). The incremental oocyte water permeability due to expression of glucose transporters was 9×10^{-4} cm/s when the incremental methylglucose permeability was 2.2×10^{-5} cm/s (Zhang et al., 1991). Assuming that the ratio of methylglucose-to-water permeabilities of the expressed and native glucose transporters are the same, the measured methylglucose permeability of J774 macrophages (3×10^{-7} cm/s) would be equiva-

lent to a glucose transporter-related water permeability for the J774 macrophage of $\sim 10^{-5}$ cm/s. This calculation suggests that the water permeability due to the glucose transporter is $\ll 1\%$ of total cell water permeability. Taken together, our results suggest that water passage through glucose transporters, although interesting from the viewpoint of structural biophysics, is quantitatively a minor contribution to cell membrane water transport. Recently, similar conclusions were reported in the red cell (Albalak, Carruthers, Grossman, and Zeidel, 1991) and in the kidney (Dempster, van Hoek, de Jong, and van Os, 1991).

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