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Evidence that the glucose transporter serves as a water channel in J774 macrophages

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ABSTRACT **Water transport across plasma membranes is a universal property of cells, but the route of such transport is unclear. In this study, volume changes of cells of the J774 murine macrophage-like cell line were monitored by recording the intensity of light scattered by the cells. We investigated the effects of several inhibitors of glucose transport on cell membrane osmotic water permeability as calculated from the rates of cell volume change. Cytochalasin B (2.5 μg/ml), phloretin (20 μM), and tomatine (3 μM) reversibly blocked glucose uptake into these cells. All three inhibitors reversibly decreased the osmotic water permeability of J774 cells from 89.6 ± 3.2 to 27.2 ± 1.4 μm/sec. We conclude that a major component of the osmotic water flow across the plasma membranes of these cells is accounted for by water traversing their glucose transporters.**

Water transport across plasma membranes is thought to occur both by diffusion across the lipid bilayer and through specific channels. Although the lipid bilayer of cell membranes has a relatively low intrinsic permeability to water, it may suffice for water exchanges in most cells (1). Some cells [i.e., urinary tract epithelial cells (2) and erythrocytes (3, 4)] display osmotic water permeabilities (P) much higher [up to 350 μm/sec (5)] than can be accounted for by osmotic water passage through lipid bilayers [P = 4–37 μm/sec (6)], suggesting that they possess membrane channels specialized for water transport. In the human erythrocyte, Solomon *et al.* (3) proposed that the anion transporter (band 3) serves as a water channel. However, other possible transmembrane water routes in this cell have been suggested (4, 7). Jung *et al.* (8) used the kinetics of hydrogen–tritium exchange in erythrocytes to suggest that the glucose transporter contains a water pore. Experimental data and theoretical analysis from others (9–11) also suggest that the dimensions and physical properties of the glucose transporter are consistent with its functioning as a water channel. Nonetheless, direct evidence identifying the water channel has been lacking.

The identification of water channels in cells has been hindered by the lack of inhibitors that selectively block water transport. We report here that a specific inhibitor of glucose transport [cytochalasin B (CytB)] (12) reversibly slows osmotic water transport across the plasma membranes of J774 cells, a murine macrophage-like cell line (13). For these experiments, we used the intensity of light scattered (I_s) by cells to monitor transient cell volume changes. From our evidence, we conclude that glucose transporters serve as the major water channel in these cells. Some of our initial results have appeared in abstract form (14).

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METHODS

Cell Culture. J774 cells were maintained as suspension cultures in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (vol/vol) heat-inactivated calf serum (GIBCO). Cells were plated and maintained overnight as adherent cultures in the above medium on either 12-mm round coverslips (for solute transport measurements), or on rectangular (20 × 6 mm) glass coverslips (for volume measurements). Final cell densities for all experiments were 3000–4000 cells per mm^2. Protein content was determined by the method of Lowry *et al.* (15).

Cell Volume Measurements. Rectangular coverslips on which J774 cells were grown were affixed to a plastic holder that formed a cap for a round glass vial (2.5 ml, total volume). Except for inlet and outlet tubing, the cap sealed the vial to form a superfusion chamber. The solution in the vial was exchanged by perfusing it rapidly (5–7 sec) with 10 ml of the desired solution. I_s was used to measure changes in the volume of J774 cells. The vials were maintained at 37°C in a dark chamber and were illuminated with a 0.5-mW He–Ne (Melles–Griot) laser beam expanded to a 7-mm diameter. The coverslips were placed at an angle of 30° with respect to the beam, with the glass surface bearing the cells facing the laser source. Light scattered at an angle of 90° to the beam was collected by a lens (2.5-cm diameter) and sent by a light guide to a photomultiplier (Hamamatsu R928). Suitable circuitry provided current to voltage conversion, filtering, and signal conditioning for a two-channel chart recorder. Degassed solutions of various osmolarities were based upon isotonic phosphate-buffered saline (PBS) containing 5.5 mM glucose in which only the concentration of NaCl was varied. For example, a 30% hypertonic solution represents an osmolarity 1.3 times greater than that of isotonic solution (290 milliosmoles). Changes in cell volume of single-cell suspensions (10^5 cells per ml) of J774 cells were monitored by measuring forward light scatter (16) with a cell sorter (FACScan, Beckton Dickinson). CytB, dihydrocytochalasin B (H_2CytB), phloretin (Phl), and tomatine (Tom) were purchased from Sigma.

Transport of Glucose and Other Substrates. The uptakes of 3-*O*-[*methyl*-^{3}H]methyl-D-glucose (MeGlc) (specific activity, 90 μCi/mmol; 1 Ci = 37 GBq), 2-deoxy[^{3}H]glucose (dGlc) (specific activity, 10 μCi/mmol), γ-[2,3-^{3}HN]aminoisobutyric acid (γAIB) (specific activity, 40 μCi/mmol), and [1-^{14}C]creatine (specific activity, 29 μCi/mmol) into adherent J774 cells were used to monitor facilitated glucose transport and γAIB and creatine uptake as described (17). Radioactive

Abbreviations: P, osmotic water permeability; I_s, intensity of scattered light; CytB, cytochalasin B; H_2CytB, dihydrocytochalasin B; MeGlc, 3-*O*-methylglucose; dGlc, 2-deoxyglucose; Phl, phloretin; Tom, tomatine; γAIB, γ-aminoisobutyric acid.

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materials were purchased from NEN, except for creatine which was from Amersham.

RESULTS AND DISCUSSION

Cell Volume by Light Scattering Determinations. In these experiments we established the validity and reproducibility of using I_s to monitor the cell volume of J774 cells adhering to glass coverslips. We measured I_s at a 90° angle. Under isotonic conditions, I_s of J774 cells remained constant for at least 2–3 hr. When the ambient osmolarity was changed, I_s changed rapidly and characteristically as a function of either increasing or decreasing the osmolarity of the superfusion solution. For example, when J774 cells were shifted from isotonic to hypotonic solutions, I_s (Fig. 1*a*) rapidly decreased (within 40 sec). This decrease in I_s reflects an osmotically induced increase in the average volume of the cells. I_s behaves as if it depends mainly on the intracellular concentration of scatterers (18).

The initial delay (10 sec) in the I_s response to an ambient osmotic shift (Fig. 1) is thought to arise partly from solution mixing and partly from an unstirred layer of solution in contact with the cells (5), which slows attainment of the imposed concentration gradient at the cell membrane boundary. The next segment of the I_s transients (initial 20–60 sec, Fig. 1) appeared exponential. The cells usually were returned to isotonic solution after some 2 min of osmotic challenge. Repeated cycles of osmotic challenge in the same cells could be carried out every 15 min for up to 3 hr without any apparent change in the pattern of the I_s response. In these and all subsequent experiments, the number of cells and the protein content per coverslip remained constant before and after osmotic challenge (data not shown).

When cells were maintained in anisotonic solutions for more than 2 min, I_s changed as if the cells were regulating their volume (19). For instance, after cells were challenged with a 10% hypotonic solution, I_s returned within 30 min to the value it had under isotonic conditions (Fig. 1*c*); on the other hand, after a 10% hypertonic challenge, I_s only readjusted back toward its former level by some 20% and then stabilized at that value (Fig. 1*d*).

Effects of Various Ambient Osmolarities on I_s and Cell Volume. When cells were exposed to a range of extracellular osmolarities from 232 to 348 milliosmoles (20% hypotonic to 20% hypertonic, respectively), I_s increased in a linear fashion (Fig. 2*a*), behaving as if it was inversely proportional to cell volume. To ensure that I_s originated from the cells, we measured I_s of cells plated on the coverslips at increasing densities. At constant (isotonic) external osmolarity, I_s increased linearly with cell density (Fig. 2*b*). This is consistent with I_s originating solely from light scattered by the cells.

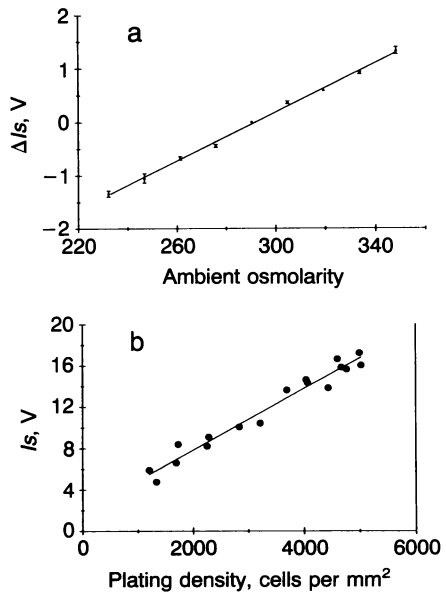

FIG. 2. (*a*) I_s changes as a function of ambient osmolarity (milliosmoles/liter). After imposing a transition from isotonic solution to the tonicity of interest, the time course of I_s was monitored and the maximum deflection (Fig. 1 *a* and *b*) was recorded. The ordinate is the difference between the final and initial I_s values. Six experiments were conducted; in each one, the cells were challenged with eight anisotonic solutions (5, 10, 15, and 20% hypotonic and hypertonic). Each point in the graph represents the mean ± SEM of the six experiments. The photomultiplier was fed −240 V; baseline I_s values averaged 13.3 ± 0.3 V (mean ± SEM). (*b*) I_s changes as a function of cell density. J774 cells plated on coverslips at the indicated densities were bathed with isotonic medium and a steady I_s value was recorded. The photomultiplier was fed −240 V. Each point in the graph represents triplicate measurements of cells on a given coverslip. A regression line was fit to the data by computer graphics (Sigmaplot). The results from 17 experiments are shown here.

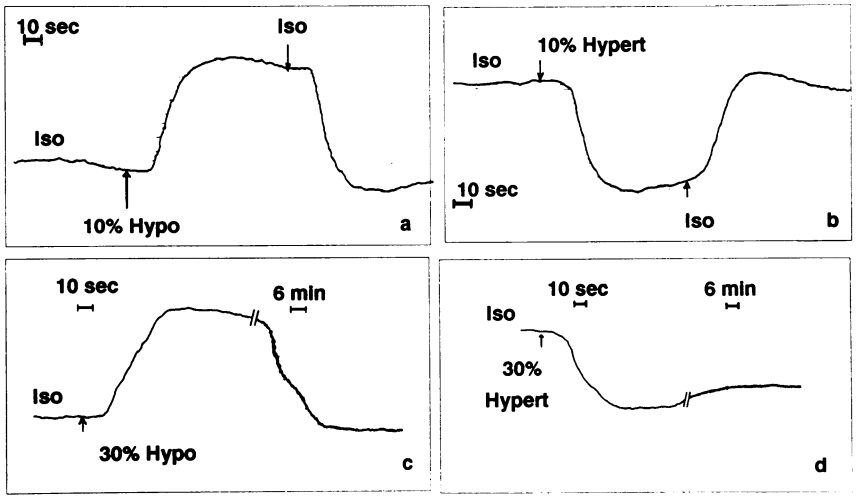

FIG. 1. Time course of I_s changes in response to osmolarity challenges. Adherent J774 cells were plated. Cells in isotonic solution were challenged with a 10% hypotonic (hypo) solution (*a*) or a 10% hypertonic (hypert) solution (*b*). Cells were subsequently returned to isotonic medium. Ordinates are I_s (arbitrary scale; I_s decreases in the upward direction). Abscissas are time. J774 cells also were maintained for longer time intervals in anisotonic solutions to observe volume regulation [30% hypotonic solution (*c*) or 30% hypertonic solution (*d*)]. The arrows mark the times of solution exchanges. Breaks in I_s denote changes in time scale. These data are representative of 40 experiments.

We employed two other methods to confirm that changes in I_s were correlated with changes in cell volume. (*i*) We measured MeGlc uptake as an independent indicator of cell volume (20). We utilized MeGlc because it is neither phosphorylated nor metabolized, and its intracellular concentration reaches equilibrium with that in the medium within 30 min (data not shown). Adherent J774 cells were bathed with either isotonic or 30% hypertonic solutions for 30 min at 37°C in the presence of labeled MeGlc. In cells bathed in hypertonic solution, the uptake of labeled MeGlc (in pmol/mg of protein) by J774 cells was only 72% of that in cells maintained under isotonic conditions [MeGlc could not be used to verify the effects of hypotonic challenge because the cells rapidly regulated their volume under hypotonic conditions (Fig. 1*c*)]. These results show that the volume into which MeGlc distributes is approximately one-third larger in cells maintained in isotonic medium than in those cells maintained in 30% hypertonic medium. This is consistent with our measurements of cell volume using optical methods.

(*ii*) We determined the volume of J774 cells maintained in suspension by measuring their I_s at low angles (forward scatter). Under these conditions, we observed (Fig. 3) that I_s increased for a given population of cells when they were subjected to hypotonic challenge and decreased in cells exposed to hypertonic medium. The changes in low-angle I_s in response to osmotic challenge for cells in suspension are consistent with the results obtained measuring 90° I_s for adherent cells, [i.e., I_s changes in response to alterations in cell volume depend on the angle at which scattered light is collected (21)]. We conclude that both suspended and plated J774 cells exhibit similar changes in volume in response to osmotic challenges.

The Effect of CytB, Phl, and Tom on dGlc Uptake by J774 Cells. CytB and Phl are well characterized inhibitors of glucose transport in a variety of cells. Therefore, we used [^{3}H]dGlc uptake by J774 cells to measure the effects of these inhibitors. CytB (2.5 μg/ml) and Phl (20 μM) inhibited the uptake of [^{3}H]dGlc by >95% and 90%, respectively, within 15 min (Fig. 4). In addition, Tom (3 μM), a glycoside closely related to digitonin, inhibited the uptake of [^{3}H]dGlc in J774 cells by >65%. All three drugs inhibited dGlc uptake in J774 cells in a dose-dependent fashion (data not shown) and had no adverse effect on cell viability (data not shown), as determined by trypan blue exclusion (17). As expected (12), H_2CytB at 2.5–5.0 μg/ml had no effect on the rate of dGlc uptake (Fig. 4) but blocked Fc receptor-mediated phagocytosis by 95% (data not shown).

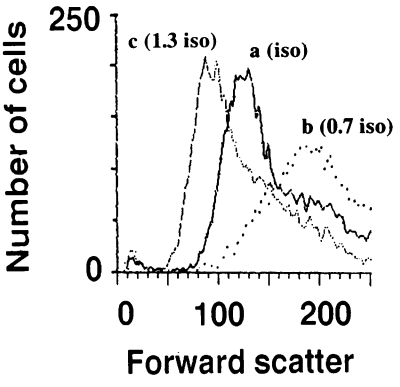

FIG. 3. Comparative volumes of J774 cells determined with a flow cytometer. The plot represents a histogram of the number of cells (ordinate) as a function of the intensity of forward scattering (abscissa, in arbitrary units). Curves a, b, and c represent cell populations bathed in isotonic (iso), 30% hypotonic (0.7 iso), and 30% hypertonic (1.3 iso) solutions, respectively; 10,000 cells were analyzed for each curve. For the anisotonic samples, cells in isotonic medium were centrifuged at room temperature at 1000 rpm for 10 min in an Eppendorf microcentrifuge, quickly resuspended in the test solutions at 37°C, and sampled within 45 sec.

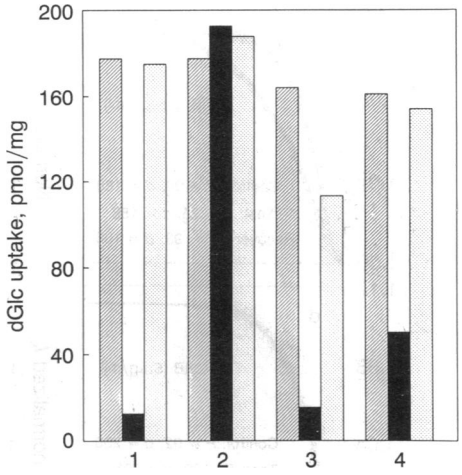

FIG. 4. Effects of CytB (bars 1), H_2CytB (bars 2), Phl (bars 3), and Tom (bars 4) on dGlc uptake in J774 cells. Cells maintained on 12-mm coverslips were incubated at 37°C in PBS in the presence or absence of CytB (2.5 μg/ml), H_2CytB (2.5 μg/ml), Phl (20 μM), or Tom (3 μM) for 5–15 min in PBS before placing the coverslips in PBS containing 0.5 μCi of dGlc in the presence or absence of the test agent. After 30 min, the uptake of dGlc was measured. Hatched bars, control cells incubated in PBS; solid bars, cells incubated with the test agent; stippled bars, cells that had been incubated with the test drug for 30 min and then washed in drug-free medium and further incubated in the absence of drug for an additional 30 min, at which time uptake of [^{3}H]dGlc was measured. Each set of bars depicts one experiment representative of three for each group. Each bar represents the average of values from three coverslips.

The inhibitory effects of CytB, Phl, and Tom on dGlc transport were rapidly reversible. For example, when CytB-treated cells were washed and maintained for an additional 15 min in drug-free PBS, the rate of dGlc uptake was comparable to that observed in untreated cells. The effects of Phl and Tom on dGlc uptake were largely reversible; cells treated with Phl or Tom for 15 min and washed with drug-free medium for 15 min took up dGlc at a rate 70–80% of that observed in untreated cells.

We also measured the effects of these compounds on other transport systems. The rates of creatine or γAIB uptake were unaffected by either CytB or H_2CytB (each at 20 μg/ml). In contrast, Tom (2 μM) and Phl (20 μM) inhibited both creatine and γAIB uptake by >90% (data not shown).

Effect of Glucose Transport Inhibitors on Cell Volume Changes Induced by Osmotic Challenge. Having established the validity of using I_s to monitor cell volume and confirmed that CytB, Phl, and Tom inhibit solute transport, we examined the effects of these inhibitors on the ability of J774 cells to respond to changes in external osmolarity. When cells were shifted from isotonic to 10% hypotonic medium in the presence of these drugs, we observed a clear decrease in the rate of I_s change by the cells (Fig. 5). All three inhibitors changed only the slope of the early transition in I_s (Fig. 5); they did not affect the maximal change in I_s in response to osmotic challenge (data not shown). Moreover, the effects of all three inhibitors were completely reversible (Fig. 5). Reversal of the effects of Phl or Tom occurred within 2 min, whereas CytB-treated cells required some 15 min to recover (data not shown). H_2CytB (5.0 μg/ml) had no effect on the rate of I_s change (Fig. 5), which suggests that cytoskeletal elements are not responsible for the inhibitory action of CytB.

The inhibitory effects of CytB, Phl, and Tom on the rate of I_s change were observed under a variety of modalities of osmotic challenge. Cells that had been challenged with drug-

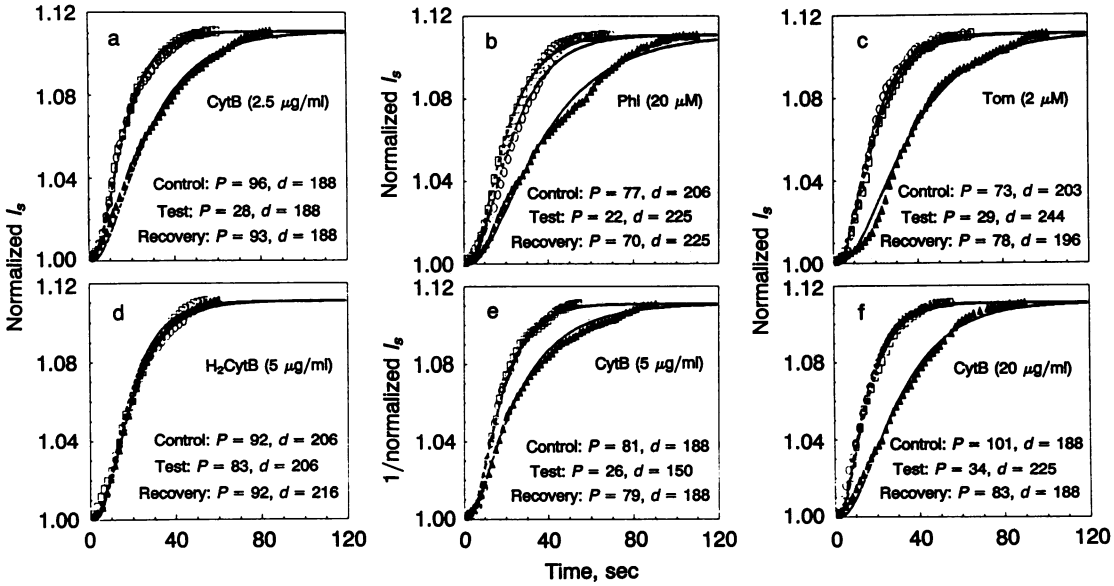

FIG. 5. Effect of osmotic challenge on I_s. Adherent J774 cells were plated. The experimental data points shown were obtained by digitizing recordings of I_s vs. t (cf. Fig. 1) with a (Jandel) tablet and a computer program (Sigmascan). Each panel shows a representative experiment in which a test compound was used at the given concentration. (*a–d* and *f*) Cells were challenged with a 10% hypotonic solution; the ordinate is normalized I_s (z). (*e*) Cells were challenged with a 10% hypertonic solution; for convenience, the ordinate here is 1/normalized I_s ($1/z$). All solutions contained 5.5 mM glucose except those used for the experiments in *f*. Three sets of data are shown that correspond to J774 cells challenged with anisotonic medium in the absence of drug (□, control), in the presence of drug (▲, test), and after washing with drug-free medium (○, recovery). The curves shown join the normalized I_s values obtained by fitting to the experimental values a solution of Eq. 1; the values of P and d with which each curve was computed are shown.

containing hypotonic solutions were then returned to isotonic solution (still in the presence of the test drug); under these conditions, all these drugs decreased the rate at which I_s returned to control values (data not shown). Similarly, CytB decreased the rate of I_s change when cells were shifted from isotonic to hypertonic solutions (Fig. 5*e*) and when cells were subsequently returned to isotonic solutions (data not shown). When glucose was omitted from the medium, J774 cells responded to an osmotic challenge at the same rate (Fig. 5*f*) as cells maintained in glucose-containing solutions. Furthermore, CytB (20 μg/ml, Fig. 5*f*, and 2.5 μg/ml, data not shown) (and not H_2CytB) dramatically decreased the rate of I_s change in response to an isotonic to 10% hypotonic transition in glucose-free medium (Fig. 5*f*) and for the subsequent return to isotonicity (data not shown). Thus, the CytB-inhibitable component of osmotically driven cell swelling and the inhibitory effect of CytB on this process do not depend on the presence of glucose in the medium.

Calculation of Osmotic Water Permeability from I_s. All changes in cell volume have been described in Figs. 1, 2, and 5 as relative changes in I_s. To translate I_s values into P values, we used a curve-fitting method (unpublished data) to calculate both P (in units of length/time), and the thickness (d) of the unstirred layer in contact with the cells. For these calculations: (*i*) the exchangeable volume of J774 cells in isotonic solution (V_0) was experimentally determined by [^{3}H]MeGlc uptake (data not shown) to be 1.8×10^3 μm^3 per cell, a volume consistent with that obtained by others (22); (*ii*) the cell surface area (A) was estimated from phase-contrast micrographs of plated cells (data not shown) to be ≈800 μm^2 per cell (23, 24); and (*iii*) the number of osmotic particles inside the cells and the cell surface area were assumed to remain constant during the osmotic transients. We solved the equation

$$dz/dt = (PV_wC_0A/V_0)[(1/z) - (C_e/C_0)] \quad [1]$$

by computer using the method of Runge-Kutta (25), where z is the ratio of the osmotically effective cell volume in hypertonic or hypotonic solutions to its osmotically effective volume in isotonic solution, V_w is the water molar volume (18 cm^3/mol), and C_0 is the osmolarity of isotonic solutions (290 milliosmoles/liter). This normalized volume z was obtained from the normalized I_s values (Fig. 5) using the data in Fig. 2*a* (i.e., maximal I_s change during the osmotic transition was in direct proportion to the percent change in osmolarity of the medium). C_e is the osmolarity immediately outside the cell membrane and is itself a function of time (t) and d, as given by Eq. 2 (26).

$$C_e = C_f + (C_0 - C_f)\left(\frac{4}{\pi}\right)\sum_{j=1}^{\infty}(-1)^j\left[\frac{1}{(2_j + 1)}\right] \times \exp\left[-\left(\frac{\pi(2_j + 1)}{2d}\right)^2 Dt\right]. \quad [2]$$

D was taken to be 1.5×10^{-5} cm^2/sec and represents the diffusion coefficient of NaCl (osmolarity differences imposed were due to changes in the concentration of NaCl in the medium); C_f is the osmolarity imposed with each challenge.

Using these equations, and the values given for A, V_0, C_0, D, and V_w, we calculated values of P and d for every data set (Fig. 6) that minimized the variance between the experimental data and the computer-generated z values for every time point (Fig. 5).

All three inhibitors of glucose transport caused a ≈70% decrease in P (Figs. 5 and 6). This decrease occurred at the same inhibitor concentration and with a similar time course and degree of reversibility as that observed when dGlc uptake was measured in the presence of these drugs and after their removal. It is important to point out that despite marked changes in P, there was no significant change in the unstirred layer d under any of the conditions examined (Fig. 6); this lends credence to the assumptions used in deriving the differential equation.

Even in the presence of glucose transport inhibitors, J774 cells still responded to osmotic challenge, but with a slower time course. From the data in Fig. 6, it appears that twice as

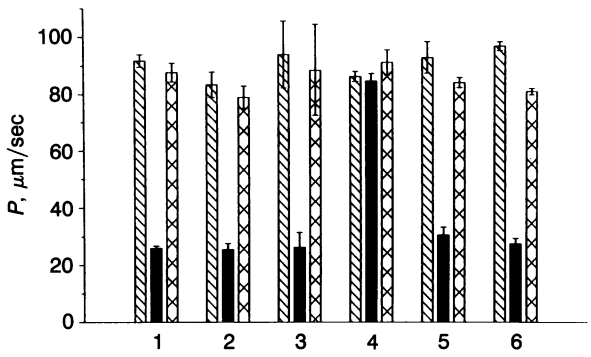

FIG. 6. *P* values. Each set of bars represents the values of permeabilities (mean ± SEM) for control (hatched bars), test (solid bars), and recovery (cross-hatched bars) conditions for the corresponding panels in Fig. 5. Drugs were used at the same concentrations as in Fig. 5. Four experiments were done in each set. Only *P* was affected by inhibitors. Values for *d* under control, test, and recovery conditions averaged for the 24 experiments were, respectively, 189 ± 5, 199 ± 9, and 192 ± 6 μm (mean ± SEM). Bars: 1, cytB (0.9 π); 2, Phl (0.9 π); 3, Tom; 4, H_2CytB (0.9 π); 5, CytB (1.1 π); 6, CytB (no glucose). π denotes isotonic concentration.

many water molecules traverse the cell membranes per unit time through the CytB-inhibitable pathway (presumably, the glucose transporter) as through the noninhibitable pathway. We hypothesize that the noninhibitable component of *P* is largely related to water crossing the lipid bilayer because the value of *P* in the presence of glucose transport inhibitors in our system (about 27 μm/sec, Fig. 6) is within the range reported for the *P* value of lipid bilayers [4–37 μm/sec, (6)]. Thus these data support our hypothesis that water traverses the plasma membrane of J774 cells through a pore in the glucose transporter.

We hypothesize that inhibitors of glucose transport are especially effective in J774 cells because of the comparatively large number of glucose transporters expressed by them. The glucose transporter translocates 10^3–10^4 molecules of glucose per sec (27). By using this value and the rate of dGlc uptake, we estimate that J774 cells express between 10^5 and 10^6 glucose transporters per cell. The fact that Tom and Phl block creatine and γ-AIB uptake yet decrease *P* to the same extent as CytB suggests that transporters for these substances make an insignificant contribution to water permeability in J774 cells.

The rate (rt_w) at which water may traverse the cell membrane through the glucose transporter (molecules per sec per transporter) is given by $rt_w = (P_p\ A\ \Delta C\ N)/n_c$, where P_p (osmotic water permeability of the protein component, 62.4 μm/sec) is the difference between the control and inhibited values of *P* (Fig. 6), *N* is Avogadro's number, Δ*C* is the osmotic gradient (taken here as 10% of the isotonic value or 29 milliosmoles/liter), and n_c is the number of glucose transporters per cell. We calculate rt_w to be 10^6–10^7 molecules per sec per transporter, which is consistent with a prior theoretical estimate of rt_w [1.4×10^6 (11)].

Although glucose transporters appear to be major conduits for water permeation in J774 cells, it is uncertain whether they play a major role in water transport in other cells. For instance, glucose permeability did not increase in antidiuretic hormone-stimulated urinary bladder even though water permeability increased (28). Bovine erythrocytes have a robust *P* [155 μm/sec (29)], yet, just as cells of many nonprimates, upon maturation they lose their ability to transport glucose (30). In human erythrocytes, Phl does not affect osmotic (31) or diffusional (32) water permeabilities. Thus, the identity of water channels in cells other than J774 macrophages remains to be elucidated. Since the interpretation of our data hinges on the specificity of CytB and on the effectiveness of Phl and Tom in inhibiting glucose transport, the involvement of the glucose transporters in water flow across cell membranes should be tested directly.

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