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Sodium-independent carrier-mediated inositol transport in cultured renal epithelial (LLC-PK$_1$) cells

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Abstract

In addition to the concentrative, Na^+-dependent inositol transport system demonstrated in many cell types, carrier-mediated, Na^+-independent inositol transport is also shown to exist in LLC-PK$_1$ renal epithelia. Inhibition of inositol uptake in Na^+-free saline by 0.1 mM phloretin, and self-inhibition by net concentrations of inositol exceeding 10 mM, demonstrate the carrier-mediation of the Na^+-independent uptake and distinguish it from flux through anion channels. The Na^+-dependent uptake exhibits higher affinity for inositol, as seen by the stronger self-inhibition at lower inositol concentrations in Na^+ saline. Kinetic analyses indicate a K_m of 178 μM and a V_{max} of 2447 pmol/min per μg DNA for the Na^+-dependent system, whereas the lower affinity, lower capacity Na^+-independent system manifests a K_m of 5.2 mM and a V_{max} of 249 pmol/min per μg DNA. The Na^+-independent uptake further differs from the Na^+-dependent transport by the lack of inhibitory effect of 10 mM glucose, and the greater relative inhibition of phloretin compared to that of phlorizin. Both types of uptake appear to localize predominantly to the basal-lateral cell surface. The Na^+-independent transport is bidirectional, functioning in efflux as well as influx of inositol.

Keywords: *myo*-Inositol; Osmolyte; Facilitated diffusion; Kidney; Phlorizin; Phloretin; Glucose; (Proximal tubule); (LLC-PK$_1$ cell)

1. Introduction

The concentrative, Na^+-dependent transport of inositol has been described in a wide variety of cell types [1–4]. Since the Na^+ electrochemical gradient is directed into the cell in normal physiological situations, this cotransport system should function only in the concentrative uptake of inositol. Na^+-independent facilitated diffusional transport can function in both efflux and influx, and unlike simple diffusion through the membrane itself, would be subject to regulation by the cell. Since inositol metabolites are integral signal transduction intermediates [5] and inositol can function in osmoregulation [3], we postulated that a facilitated diffusional inositol transport system would exist. Possible functions for such a transport system would be regulation of intracellular inositol concentration and/or renal inositol reabsorption. This transport activity, via a unique facilitated diffusional transport system, has been reported in yeast [6] and non-mammalian intestine [7]. We would speculate that many studies exclude a Na^+-independent inositol transporter because uptake vs. concentration curves for this substrate are normally not performed beyond 5 mM extracellular inositol concentrations. At this concentration, saturability would not be in evidence, and the wrong conclusion could be formed.

The LLC-PK$_1$ pig kidney epithelial cell line has become a widely studied model for renal proximal tubular and general epithelial transport activity such as glucose uptake [8], phosphate transport [9], Na^+/H^+ exchange [10], and calcium uptake [11]. At confluence these cells form a polar epithelium which permits not only the study of intracellular inositol uptake and efflux, but, the polar localization of inositol transport systems on either the apical or basal-lateral cell membrane.

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2. Materials and methods

2.1. Cell line

LLC-PK_1 cells were a gift of Dr. R.N. Hull [12] and were used between passage levels 185 and 200. Cells were routinely passaged weekly by seeding $1 \cdot 10^5$ cells into a 75 cm^2 culture flask (Falcon) containing 25 ml of α-minimum essential medium without nucleosides (JRH Biosciences) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine. Cells were suspended for these seedings by rinsing confluent monolayers three times with Ca^{2+}/Mg^{2+}-free PBS (JRH Biosciences) and incubating at 37° C with 0.25% trypsin/0.02% EDTA (Gibco) until cells detached. Mycoplasma tests based on immunofluorescent assays for Mycoplasma DNA using Hoechst dye [13] were performed every 8–10 weeks and were consistently negative. For routine transport studies, cells were seeded at confluent density into a 100-mm petri dish containing seven 25 mm Nuclepore filters (0.8 μm pore) that had been previously collagen-coated (Ethicon) and affixed to the dish. For uptake studies from specifically either the apical or basal-lateral cell surface, cells were seeded at confluent density into 30 mm Falcon 3090 filter cup assemblies. These filter cups do not require collagen coating. Three such filter-cups containing 2 ml of cell suspension were situated in 100-mm petri dishes with 15 ml of culture medium.

2.2. Uptake studies

Confluent cell sheets on 25 mm collagen-coated Nuclepore filters were removed from the 100 mm culture dish and rinsed three times in 22° C, 3-(*N*-morpholino)propane sulfonic acid (Mops)-buffered saline [8], then added to a 35 mm petri dish containing 2 ml of this saline with the indicated concentration of *myo*-[2-^{3}H(N)]inositol (1–2 μCi/ml) (DuPont NEN; 52.4 Ci/mmol) and 0.1 mM [^{14}C]mannitol (1–2 μCi/ml) (Dupont NEN; 55 mCi/mmol). The osmolality of all salines was checked prior to the experiment and determined to be 300 ± 6 mosmol/kg water. Incubations were conducted at 22° C on an orbital shaker, with cell sheets immersed in this saline thereby allowing access to apical and basal-lateral cell surface. Cell sheets were then removed from the incubation saline and passed through five 4° C saline rinses with 0.1 mM phloretin. Cell sheets were then placed in 0.4 M perchloric acid to lyse cells and precipitate DNA. Samples of supernatant were taken for dual label liquid scintillation counting, the D-[^{14}C]mannitol counts being used to determine the percentage of [^{3}H]inositol counts which were not intracellular. After washing the perchloric acid precipitate with 0.4 M perchloric acid, the precipitated DNA was hydrolyzed in 1 M perchloric acid at 78° C for 45 min. DNA was then assayed spectrophotometrically [14]. Results were calculated as the dpm of [^{3}H]inositol taken into the cells per μg of DNA. For kinetic analyses in Na^+ or Na^+-free saline (wherein Na^+ is replaced by choline) uptakes of a wide range of inositol concentrations were determined, each at several time points. Uptakes were performed with cell sheets on 25 mm filters as described above. Rates of uptake (pmol/min per μg DNA) were determined from the regression slope of the linear portion of these plots of uptake vs. time. For inhibition analyses, a two-tailed test of significance was applied [15].

When uptake from specifically the apical or the basal-lateral membrane was to be determined, cell sheets were cultured in Falcon 3090 filter-cup assemblies as discussed above. Intactness of the cell sheets was determined by measuring transepithelial resistance of approx. 200 ohms cm^2 with 40 μA direct current pulses as described previously [16]. Saline labeled with [^{3}H]inositol and [^{14}C]mannitol, as above, was added to either the apical or the basal-lateral compartment, with unlabeled saline in the opposite fluid compartment. After incubation, the cell sheet was cut from the filter-cup with a scalpel, and analyses of intracellular inositol and DNA were performed as described above.

2.3. Efflux studies

Confluent cell sheets were loaded (90 min) in Na^+ saline with various concentrations of [^{3}H]inositol as described above. After this uptake period, one set of cell sheets (control) was rinsed five times in 4° C Na^+-free saline and analyzed for total dpm per ml cell water, and the percentage of dpm existing as free inositol (see below). The remaining sets of cell sheets were placed in Na^+ or Na^+-free saline at 22° C with or without inhibitors of efflux, on a rotator for 30 min. Cell sheets were then removed, rinsed five times in 4° C Na^+-free saline, and analyzed as described above for dpm and DNA.

2.4. Chromatographic analyses

A portion of the control cell sheets described above was added to centrifuge tubes containing boiling water, and then boiled for an additional 10 min. After vortexing and centrifuging, the supernatant was evaporated to dryness under vacuum (Savant Speed Vac), redissolved in water, and analyzed by thin-layer chromatography. Cellulose TLC plates (Analtech) and a 4:1 isopropanol/water solvent system were used. Plates were then scanned by the Bioscan 2000 system, using a [^{3}H]inositol standard.

2.5. Measurement of intracellular total inositol

Cultures of LLC-PK_1 were grown to confluence in 100 mm tissue culture dishes (Falcon). Confluent cultures were rinsed two times with cold PBS and extracted with 0.6 M TCA. The extract was neutralized after five washes with water-saturated ether (1 vol. extract/2 vol. diethyl ether).

Table 1
Uptake of 25 μM [^{3}H]inositol in the presence and the absence of Na^+

	Inositol uptake			
	Na^+-saline (dpm/μg DNA/15 min)		choline saline (dpm/μg DNA/30 min)	
Control	260 ± 20	–	32 ± 6	–
+5 mM inositol	5 ± 1	$P < 0.01$	55 ± 10	N.S.
+0.1 mM phlorizin	87 ± 5	$P < 0.05$	55 ± 12	N.S.

Values shown represent the means ± S.E. for $n = 3$–5 determinations. This experiment was repeated with cells of a later passage, and similar results were obtained. N.S. indicates not significant.

The *myo*-inositol content of the neutralized tissue extract was determined spectrophotometrically as described by MacGregor and Matschinsky [17].

3. Results

Investigations of inositol transport in various cell types generally utilize an inositol concentration similar to that normally found in human plasma, i.e., 20 μM to 80 μM [2]. As shown in Table 1, if LLC-PK_1 cells are incubated in a Na^+-saline containing 25 μM inositol, the carrier-mediated uptake is readily apparent by the significant inhibition exerted by the glycoside phlorizin, as well as the self-inhibition by inositol itself. This represents a manifestation of the general phenomena of competition and saturability in carrier-mediated transport, phenomena which cannot be present in simple diffusional uptake [18]. There is correspondingly an *apparent* lack of carrier mediation for 25 μM inositol uptake in a Na^+-free saline, namely no inhibitory effect of phlorizin nor any self-inhibition by 5 mM inositol (Table 1).

However, since intracellular inositol levels are generally in the millimolar range [19,20], and LLC-PK_1 cells were found to contain a free inositol concentration of 8.7 ± 0.6 mM, we postulated that the more significant comparison

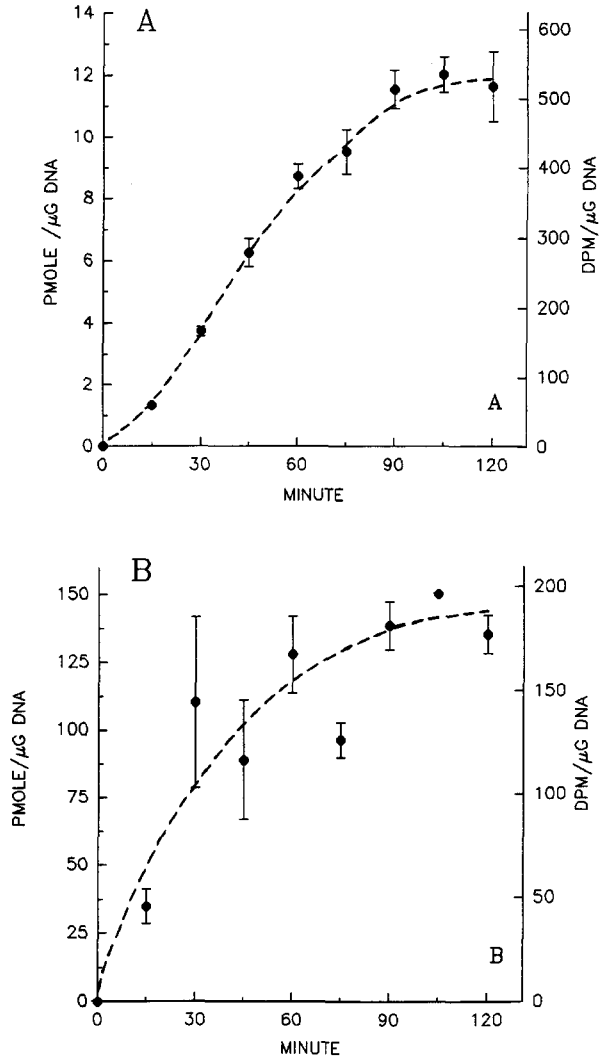

Fig. 1. Time-courses of 25 μM inositol uptake in Na^+ saline (A) and 5 mM inositol uptake in Na^+-free saline (B) by confluent LLC-PK_1 cell sheets. As described in Materials and methods, cell sheets were incubated for up to 120 min at 22° C in either Na^+ or Na^+-free radiolabeled saline, and processed for dpm and DNA. Values shown represent the means ± S.E. for $n = 6$ determinations. These uptakes were repeated with cells of a later passage, and similar time-courses were obtained.

Table 2
Inhibition of the uptake of 25 μM [^{3}H]inositol in Na^+-saline vs. 5 mM [^{3}H]inositol in Na^+-free saline

	Inositol uptake			
	Na^+-saline ($n = 7$) (dpm/μg DNA/15 min)		choline saline ($n = 6$) (dpm/μg DNA/30 min)	
Control	494 ± 4	–	56 ± 4	–
+5 mM inositol	5 ± 5	$P < 0.001$	36 ± 2	$P < 0.01$
+40 mM inositol	N.M.	–	6 ± 3	$P < 0.001$
+10 mM glucose	233 ± 40	$P < 0.01$	64 ± 3	N.S.
+0.1 mM phlorizin	209 ± 13	$P < 0.01$	62 ± 3	N.S.
+0.1 mM phloretin	322 ± 7	$P < 0.01$	41 ± 5	$P < 0.05$

As described in Materials and methods, confluent LLC-PK_1 cell sheets on collagen-coated filters were incubated in the appropriate radiolabeled saline for 15 min at 22° C with or without the indicated inhibitor. Values shown represent the means ± S.E. The experiment was repeated with cells of a later passage and similar results were obtained. N.M. indicates not measured. N.S. indicates not significant.

Table 3
The concentrative accumulation of [^{3}H]inositol within LLC-PK$_1$ in the presence of Na^+

	[^{3}H]Inositol activity in saline (dpm/ml)	Total intracellular ^{3}H (dpm/μg DNA)	Intracellular *free* [^{3}H]inositol (dpm/μg DNA)	Intracellular *free* [^{3}H]inositol activity (dpm/ml cell water)	S_i/S_o
Na^+ saline (25 μM inositol)	$1.24 \cdot 10^6$ ($\pm 1.6 \cdot 10^4$)	1119 ± 52	1001 ± 47	$1.0 \cdot 10^7$ ($\pm 0.1 \cdot 10^7$)	8.23 ±0.39
Choline saline (5 mM inositol)	$7.37 \cdot 10^6$ ($\pm 3.6 \cdot 10^4$)	99 ± 2	15 ± 0.4	$1.6 \cdot 10^5$ ($\pm 0.4 \cdot 10^4$)	0.021 ± 0.001

As described in Materials and methods, confluent LLC-PK$_1$ cell sheets on collagen coated filters were incubated in the appropriate radiolabeled saline supplemented with either 25 μM or 5 mM inositol as indicated for 90 min. Intracellular free [^{3}H]inositol was determined by thin-layer radiochromatography. The data are presented as the means (±S.E.; $n = 4$). The accumulation of dpm of [^{3}H]inositol per ml cell water is derived by dividing dpm (free [^{3}H]inositol)/μg DNA by $0.98 \cdot 10^{-4}$ ml cell water/μg DNA [8]. S_i/S_o is the ratio of the intracellular (free) inositol activity divided by the extracellular (incubation saline) inositol activity. Because the radiochromatography would not account for that amount of [^{3}H]inositol metabolized by inositol oxygenase with subsequent generation of ^3H_2O, no conclusions should be drawn from this data concerning different metabolic fates of inositol following transport via Na^+-dependent vs. Na^+-independent pathways.

would be *micro*molar inositol uptake in Na^+-saline vs. *milli*molar inositol uptake in Na^+-free saline. Although an inositol influx pathway would therefore require affinity only in the μM range, an inositol efflux system (bidirectional) would require an affinity in the mM range. When we first compare simply the time courses of 25 μM inositol uptake in Na^+-saline and 5 mM inositol uptake in Na^+-free saline (Fig. 1), both are near linear over the first 75 min but then both approach steady state by approx. 90 min and clearly plateau by 120 min. Since all [^{3}H]inositol uptakes are performed as dual-label studies with D-[^{14}C]mannitol also present in the incubation saline (see Materials and methods), the uptakes shown here are at least partially corrected for simple diffusional uptake of inositol. Routinely a percentage of [^{3}H]inositol dpm are subtracted from the total, based upon the amount of [^{14}C]mannitol dpm in the cell sheet after incubation and rinsings. This percentage is high (occasionally greater than 90%) for Na^+-free incubations of short duration (less than 15 min) and low (less than 10%) for uptakes in Na^+-saline of durations of 5 min or longer.

The carrier-mediated nature of the Na^+-independent uptake of inositol can be seen in the data of Table 2. When 5 mM extracellular [^{3}H]inositol in Na^+-free saline is self inhibited by an additional 5 mM inositol, a significant decrease (36% inhibition) of uptake is observed. 40 mM inositol inhibits the uptake of 5 mM [^{3}H]inositol (in Na^+-free saline) to an even greater extent (90% inhibition). This contrasts with the lack of significant inhibition of Na^+-independent 25 μM [^{3}H]inositol uptake by 5 mM inositol as shown in Table 1, a frequently applied test purported to show the lack of carrier mediation (in Na^+-free saline) in many published studies. The key difference between the conditions of Tables 1 and 2 is simply that in Table 2 the *total* concentration of extracellular inositol is raised well above 5 mM, a concentration range in which saturability will be evidenced. Dilution of isotope cannot by itself explain this decreased number of ^{3}H dpm in the cells in the presence of added unlabeled inositol in Na^+-free saline. If entry of [^{3}H]inositol in Na^+-free saline was achieved by simple diffusion, the rate of entry of labeled inositol would not be affected by any amount of unlabeled inositol. Only if in the presence of Na^+-free saline is there competition between labeled and unlabeled inositol for entry into the cell at a finite number of sites (transporters) could a decreased number of intracellular ^{3}H dpm actually result.

In Na^+-saline (Table 2), glucose (52% inhibition) and phlorizin (58% inhibition) both significantly inhibit inositol uptake as shown earlier for the kidney [25]. The

Table 4
Na^+-independent efflux of inositol by LLC-PK$_1$ cells

Condition	Total dpm/10^6 cells after efflux period	% of total (initial) dpm	dpm *free* inositol/10^6 cells after efflux period		% of *free* inositol (initial) dpm/10^6 cells
Control (no efflux)	(1446 ± 97)	(100) [a]	(241 ± 38)	–	(100) [b]
Efflux in choline saline	1259 ± 55	87	54 ± 9	$P < 0.01$	22
Efflux in choline saline with 0.1 mM phloretin	1375 ± 43	95	107 ± 28	$P > 0.05$	44

Cell sheets were incubated 90 min at 22° C in Na^+-saline containing 40 mM [^{3}H]inositol, passed through five rapid rinses in 22° C choline saline, then placed for 30 min on a rotator in 2 ml of 22° C choline saline with or without 0.1 mM phloretin. Cell sheets were then processed for determination of intracellular radioactivity as described in Materials and methods. Control cell sheets were simply rinsed five times at 4° C after the loading period, and intracellular radioactivity was determined. Values shown represent the means for $n = 4$ cell sheets ± S.E. This experiment was repeated with cells of a later passage and similar results were obtained.

[a] This includes [^{3}H]inositol metabolites which would not be available for efflux (as determined by TLC of the control condition) and represented 83.5% of total dpm.

[b] This is a reflection of the relative amount of *free* inositol at $t = 0$ (i.e., available for efflux at the start of the 30 min period). It does not reflect distributions of radiolabel at $t = 30$ min, since some further metabolism may have occurred even at 22° C.

Na^+-independent uptake system shows the exact opposite profile, namely an insignificant effect by glucose and a 27% inhibition by phloretin, which contrasts phlorizin's lack of significant inhibition. The inhibitory profiles by phlorizin, phloretin, and glucose on inositol transport in the presence versus the absence of Na^+ are therefore distinct from each other.

A comparison of intracellular dpm (corrected as described above for the percentage of intracellular radioactivity which exists as free [^{3}H]inositol) vs. extracellular radioactivity in the incubation saline illustrates the concentrative vs. non-concentrative nature of inositol transport in Na^+ vs. Na^+-free saline, respectively (Table 3). Specifically, the intracellular radioactivity expressed as dpm/μg DNA can be multiplied by that percentage of intracellular radioactivity which thin-layer chromatography shows to be free [^{3}H]inositol, thereby excluding those dpm which are inositol metabolites. This revised dpm (free inositol)/μg DNA is then divided by the previously described ratio of LLC-PK$_1$ cell water to DNA ($0.98 \cdot 10^{-4}$ ml H_2O_i/μg DNA; [8]) to yield an intracellular activity value of dpm/ml H_2O_i. This intracellular activity can then be compared to the extracellular activity of the saline as the ratio S_i/S_o. As seen in Table 3, this ratio is substantially greater than 1.0 in a Na^+ saline, and less than 1.0 in a Na^+-free saline. This indicates accumulation of radiolabeled free inositol in the presence of Na^+, and the inability (after 90 min) to accumulate in the absence of Na^+, suggesting concentrative transport operating in Na^+ saline but not in Na^+-free saline. If one assumes steady state conditions (intracellular and extracellular [^{3}H]inositol specific activities being equal) then the activities reported in Table 3 equate to intracellular inositol concentrations of 0.3 mM for 25 μM extracellular inositol in Na^+ saline, and 0.2 mM for 5 mM extracellular inositol in Na^+-free saline.

To determine kinetic constants for Na^+-dependent and Na^+-independent inositol uptake, initial velocities of uptake were determined for a wide range of inositol concentrations in Na^+ saline and Na^+-free saline where Na^+ was replaced by choline. Each reported velocity in these graphs is determined from the slope of the linear regression of amount of uptake vs. time for three time points of inositol uptake at each inositol concentration. Simple diffusional

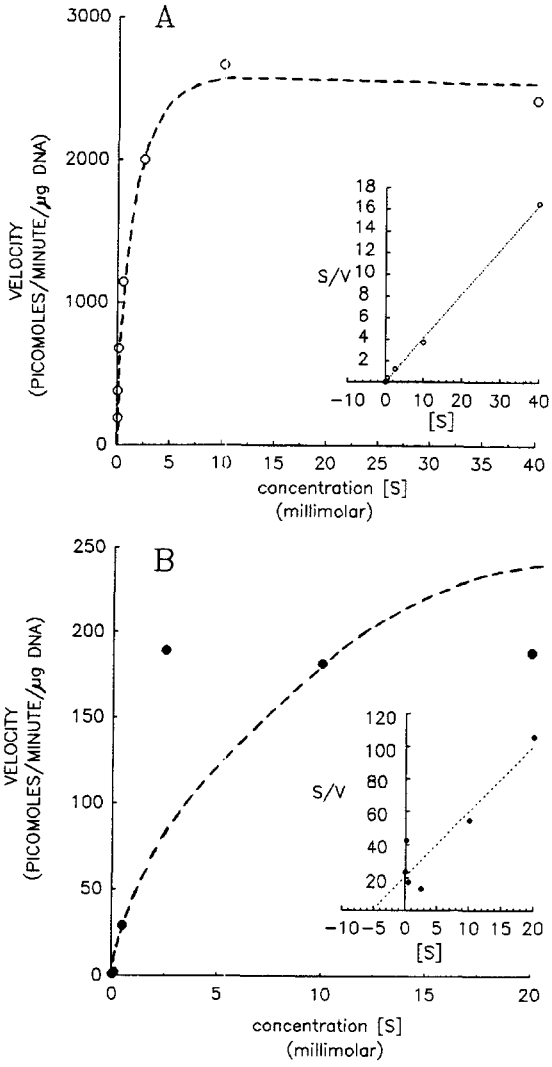

Fig. 2. Velocity vs. substrate concentration for Na^+-dependent and Na^+-independent inositol uptake by confluent LLC-PK$_1$ cell sheets. Uptake rates (pmol/min per μg DNA) are plotted as a function of inositol concentration in Na^+ (A) or Na^+-free saline (B). Uptake rates are determined as described in Materials and methods and then corrected for uptake due to simple diffusion by prior calculation of the K_d for uptake (due to simple diffusion) at saturating levels of substrate. The curves in both panels are constructed based upon prior determination of K_m and V_{max} by S/v_o vs. S plots (see inset), thereby establishing the curves' rate of rise and extent of rise, respectively. The two insets in this figure show plots of the uptake rate data of Fig. 2 according to the form $S/v = (1/V_{max})[S] + K_m/V_{max}$ where the K_m becomes the absolute value of the x intercept and the V_{max} can be obtained from the reciprocal of the slope.

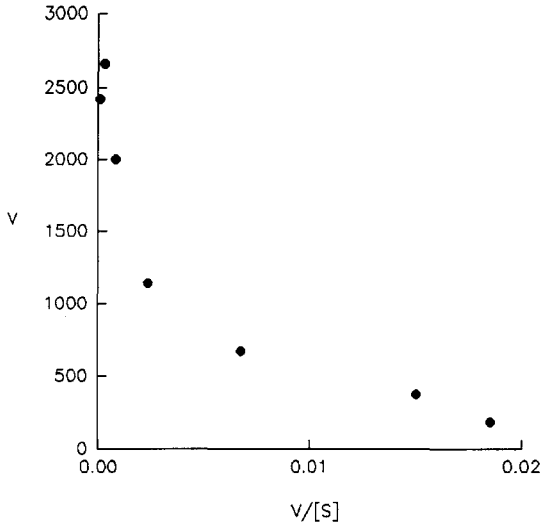

Fig. 3. Eadie-Hofstee plot of the Na^+-dependent uptake data. V vs. v/S plot of the inositol uptake data in the presence of Na^+ shown in Fig. 2A.

uptake of inositol (beyond that accounted for by dual label uptakes with [^{14}C]mannitol) was determined by measuring the velocity of uptake at saturating substrate concentrations of inositol (in excess of 10 mM), the slope of this line providing the K_d for simple diffusion. After correcting the net uptake data for uptake due to simple diffusion, this carrier-mediated uptake was plotted as a function of substrate concentration as shown in Fig. 2A and B. In panel A the uptake in the presence of Na^+ can be seen to plateau at a velocity of approx. 2500 pmol/min per μg DNA. In panel B, uptake velocities in the absence of Na^+ are shown. In this case, the rise to the plateau value is less rapid, and the plateau is significantly lower, only approx. 200 pmol/min per μg DNA, suggesting a lower capacity system.

The insets to Fig. 2 replot the data in the form of S/v vs. S, a plot in which the x intercept equates to $-K_m$ and V_{max} is approximated by the inverse of the slope of the line, this type of plot being less affected by any contribution of simple diffusion to total uptake [21]. From computer-generated linear plots of these data, the intercepts and slopes yielded a K_m of 178 μM and a V_{max} of 2447 pmol/min per μg DNA for Na^+-dependent uptake, and a relatively low affinity, low capacity, Na^+-independent uptake system of 5.2 mM, K_m, and a V_{max} of 249 pmol/min per μg DNA. The degree and pattern of scatter specifically in the low concentration data of Fig. 2B may be evidence of cooperativity and/or an additional Na^+-independent transport system. For the present study, we however assume one (Na^+ -independent) system for the purposes of kinetic derivations. These kinetic data would also be affected by compartmentalization of cellular inositol, which Berry and coworkers [22] showed to exist in hepatocytes, and which would explain apparent kinetic anomalies of inositol uptake and metabolism.

An Eadie-Hofstee plot (v vs. v/S) of the uptake velocities in the presence of Na^+ (where both the Na^+-dependent and Na^+-independent systems would be operative) provides additional evidence for the presence of two distinct inositol transporters (Fig. 3). Unlike the typical linear plot which would be indicative of a single transport system, our results yielded a curvilinear plot where uptake proceeds by a high capacity, high affinity system at very low substrate concentrations, and then would shift somewhat to a lower capacity, lower affinity system at progressively higher substrate concentrations.

As seen in Table 4, the Na^+-independent, phloretin-inhibitable uptake system functions in efflux from the cell as well as uptake into the cell, a result consistent with the general bidirectional nature of Na^+-independent facilitated-diffusional transport. After a 90 min uptake of 40 mM [^{3}H]inositol in Na^+ saline (22° C), a 30 min efflux in Na^+-free saline (22° C) results in the loss of only 13% of total initial intracellular dpm. However, chromatographic analyses of intracellular radioactivity at the end of the uptake period (as in Table 4) showed that only 16% of intracellular dpm existed as free inositol. Therefore, considering only the *free* intracellular radiolabeled inositol, approx. 75% was effluxed. The presence of 0.1 mM phloretin in the efflux saline significantly inhibited the inositol efflux. Instead of losing 75% of intracellular free [^{3}H]inositol, only 30% was lost with phloretin present in the extracellular saline.

To determine if the Na^+-dependent and Na^+-independent transport systems are localized on different cell surfaces, the uptake of 25 μM inositol (in Na^+-saline) and 5 mM inositol (in Na^+-free saline) were compared from the apical vs. basal-lateral fluid compartment (Table 5). In both cases the uptake was markedly greater from the basal-lateral side, suggesting that both the Na^+-dependent and Na^+-independent inositol transport activity is located, at least predominantly, in the basal-lateral membrane. The amount of uptake which is seen when [^{3}H]inositol is presented to the apical side (and the fact that it is inhibitable) may be due to inositol in the apical compartment passing between cells (paracellularly through the tight junction) and entering the cell at the basal-lateral membrane, as was shown to be true for glucose analogs [33], or

Table 5
Apical vs. basal-lateral uptake of 25 μM inositol in Na^+-saline and 5 mM inositol in Na^+ free saline

	Inositol uptake (dpm [^{3}H]inositol/μg DNA/15 min)			
	apical		basal-lateral	
Na^+-saline				
25 μM inositol (Control)	157 ±11	–	541 ±37	–
+0.5 mM phlorizin	21.4 ± 3.1	$P < 0.01$	128 ±13	$P < 0.01$
+5.0 mM inositol	2.1 ± 1.0	$P < 0.01$	8.1 ± 2.7	$P < 0.01$
Na^+-free saline				
5 mM inositol (Control)	34.2 ± 1.1	–	226 ± 9	–
+0.5 mM phloretin	2.5 ± 0.8	$P < 0.01$	100 ±12	$P < 0.01$
+40 mM inositol	11.9 ± 1.0	$P < 0.01$	85.1 ± 4.0	$P < 0.01$

Values shown represent the means ± S.E. for $n = 3$ cell sheets. The experiment was repeated with cells of a later passage, and similar results were obtained.

due to imperfect polar localization of inositol transporters, as we observed for Na^+,K^+-ATPase in these same cells [23].

4. Discussion

Our data indicate that LLC-PK_1 cells possess not only carrier-mediated Na^+-dependent but also carrier-mediated Na^+-independent inositol transport activity. The bases for this finding on Na^+-independent uptake are: (1) self-inhibition of uptake at extracellular inositol concentrations exceeding 5 mM; (2) inhibition of this uptake by phloretin (but not by phlorizin); and (3) the appearance of two transport systems in plots of initial velocities of uptake vs. inositol concentration, one being high affinity, high capacity, the other being low affinity, low capacity. The saturability and phloretin sensitivity of this Na^+-independent system in LLC-PK_1 cells fully distinguish it from the inositol flux via anion channels reported for glial cells [24].

There are several observed dissimilarities between Na^+-independent and Na^+-dependent inositol uptake. First, self-inhibition of Na^+-independent uptake occurs in the mM range of concentration rather than the μM range. As seen in Table 1, attempting to inhibit 25 μM inositol uptake in a Na^+-saline with 5 mM inositol is highly effective (greater than 95% inhibition), but in a Na^+-free saline it is ineffective. This could erroneously lead to the assumption that Na^+-independent uptake in these cells is not carrier mediated. However, if unlike the high affinity, μM range K_m of the Na^+-dependent system [3,4], the Na^+-independent transporter is relatively low affinity, then self-inhibition of this system will not be readily manifested until the net extracellular inositol concentration is brought sufficiently above the K_m (thereby no longer in the linear portion of a V vs. S relation). If the K_m is of the order of reported *intracellular* inositol levels of 5–10 mM [19,20], then self-inhibition will be manifested only if this concentration is exceeded, as seen in Table 2. Our kinetic data indicate a relatively low affinity (5.2 mM K_m) Na^+-independent inositol transporter, vs. a 178 μM K_m transport system in the presence of Na^+. A second dissimilarity is the widely different capacities of the two transport systems, namely V_{max} values of 2447 vs. 249 pmol/min per μg DNA in the presence and absence of Na^+ respectively.

A third dissimilarity between the Na^+-dependent and Na^+-independent transport is that Na^+-independent uptake appears not to be concentrative. After 90 min at 22° C in a Na^+-saline with 25 μM [^{3}H]inositol, the LLC-PK_1 cells achieve an intracellular free [^{3}H]inositol activity of $1.9 \cdot 10^6$ dpm/ml cell water which equates to an intracellular/extracellular concentration gradient of 8.23, expressed as S_i/S_o (Table 3). However, 90 min in a Na^+-free saline with 5 mM [^{3}H]inositol yields a gradient (S_i/S_o) of free [^{3}H]inositol of only 0.02.

A fourth dissimilarity is the different patterns of inhibition by glucose, phlorizin and phloretin. Whereas Na^+-independent inositol uptake is not significantly inhibited by glucose, and phloretin is a more potent inhibitor than phlorizin, Na^+-dependent inositol uptake is inhibited by glucose, and phlorizin is more potent than phloretin. This inhibition by glucose and phlorizin of Na^+-dependent inositol uptake has been observed in many preparations [1,25,27]. The description of a Na^+-independent, basal-lateral inositol transporter in eel intestine similarly showed inhibition by phloretin but not by phlorizin, but this system was inhibited by glucose and was relatively high affinity [26].

Although another dissimilarity was expected in the polar localization of these two transport systems, both the Na^+-dependent and Na^+-independent inositol transport activity localized to the basal-lateral cell surface of LLC-PK_1 cells. This is in contrast to the situation for LLC-PK_1 glucose transport, where we previously observed that the Na^+-dependent transport system is apical, and the Na^+-independent transport system is basal-lateral [28]. It also contrasts with the Na^+-dependent and Na^+-independent inositol transporters of eel intestine which were also respectively apical and basal-lateral [7,26]. However, the Na^+-dependent inositol transporter of MDCK renal epithelia was found to be basal-lateral [29].

As discussed by Baxter et al. [1], the Na^+-dependent inositol transporter is present on almost all cell types, differing therein from the Na^+-dependent glucose transporter, present chiefly on epithelia engaged in reabsorption. The Na^+-dependent inositol transporter's role is apparently concentrative, namely to raise intracellular inositol to mM levels from the μM levels found in plasma [2], since plasma inositol is the main source of cellular inositol for many cell types. Considering the essential roles played by inositol as a non-ionic osmolyte [3] and by inositol-metabolites in signal transduction [5], we would speculate from our studies that a Na^+-independent inositol transporter would be functional in intracellular inositol homeostasis. Being Na^+-independent it will function in both efflux and influx as shown in Table 4. Phlorizin-sensitive inositol efflux (which also manifests counter-transport) has recently been shown to occur in hepatocytes [22]. Being a low affinity system, it would essentially have little function until the high affinity, Na^+-dependent system elevates cellular inositol levels to a certain range. This range of intracellular inositol concentration will then be a function of *both* the Na^+-dependent and Na^+-independent inositol carriers. A low capacity, low affinity efflux system operating in tandem with a high capacity, high affinity influx system (as shown by our kinetic data) would be expected for cells to generate the high inositol concentration gradients from *micro*molar levels in serum to *milli*molar levels in cells.

Regulation of the Na^+-independent system as a function of extracellular osmolarity will be the subject of future work since the contribution (capacity) of this relatively

low affinity system may increase under certain physiological conditions. Recent findings that extracellular osmolarity will regulate the number of Na^+-dependent inositol transporters, and thereby the cells' inositol concentrative capacity [24,30–32] attest the overall interest in this area. We submit that the cell's regulatory capacity may extend to Na^+-independent inositol transporters as well, with the Na^+-independent system functioning as a 'safety-valve' against excessively high intracellular inositol levels under certain physiological conditions.

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