Biochimica et Biophysica Acta 857 (1986) 198–208
Elsevier

BBA 73084

Non-competitive inhibition of *myo*-inositol transport in cultured bovine retinal capillary pericytes by glucose and reversal by Sorbinil

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(Received March 13th, 1985)
(Revised manuscript received January 30th, 1986)

Key words: *myo*-Inositol; Glucose; Transport; Retinal capillary pericyte; Diabetes

***myo*-Inositol transport by retinal capillary pericytes in culture was characterized. The major *myo*-inositol transport process was sodium-dependent, ouabain-sensitive, and saturable at 40 mM, indicating a carrier-mediated process. The sodium ion concentration required to produce one-half the maximal rate of *myo*-inositol uptake ($[Na^+]_{0.5}$) did not show dependence on the external *myo*-inositol concentration (22.3 mM sodium for 0.005 mM *myo*-inositol; 18.2 mM sodium for 0.05 mM *myo*-inositol). *myo*-Inositol transport was an energy-dependent, active process functioning against a *myo*-inositol concentration gradient. The kinetics of the sodium-dependent system fitted a 'velocity type' co-transport model where binding of sodium ion to the carrier increased the velocity (V_{max} 28 to 313 pmol *myo*-inositol/μg DNA per 20 min when $[Na^+]$ varied from 9 to 150 mM) but not the affinity for *myo*-inositol (K_m 0.92 to 0.83 mM when $[Na^+]$ varied from 9 to 150 mM). Metabolizable hexoses (D-glucose or D-galactose; greater than 5 mM) inhibited *myo*-inositol uptake. Dixon-plot analysis indicated that the inhibition was non-competitive with a K_i of 22.7 mM for D-glucose and 72.6 mM for D-galactose. The inhibition was significantly reversed by Sorbinil (0.1 mM), an aldose reductase inhibitor. In contrast, high concentrations of non-metabolizable hexoses (L-glucose, 3-*O*-methyl-D-glucose), or partially metabolizable 2-deoxy-D-glucose, did not significantly inhibit *myo*-inositol uptake. The inhibitory effect of D-glucose or D-galactose on *myo*-inositol transport appeared to be related to glucose or galactose metabolism via the polyol pathway.**

Introduction

Hyperglycemia per se may play an etiological role in diabetic microangiopathy [1–6]. A loss of pericytes is an early characteristic of diabetic microvascular disease. In order to understand the relationship between hyperglycemia and early functional and structural alterations of retinal microvessels in diabetic retinopathy, the effects of glucose on the metabolism, cellular function and structure of retinal capillary pericytes are being studied in culture [2–7].

High concentrations of glucose have been reported to inhibit the uptake of *myo*-inositol by nerves and glomeruli in diabetic animals [8–13], and by endoneurial preparations and Schwann cells in vitro [10,14,15]. Reduced neural *myo*-inositol levels were accompanied by elevated cellular sorbitol and decreased sodium-potassium ATPase activity levels [12]. These changes were reversed by Sorbinil [12,13]. Functional neural abnormalities in diabetic animals, occurring in the presence of increased neural sorbitol and decreased neural *myo*-inositol, also could be prevented with Sorbinil

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[12]. When a fall in nerve *myo*-inositol was prevented in vivo by oral *myo*-inositol administration, the nerve conduction velocity change was prevented, even though the increased sorbitol in the nerve was not suppressed, suggesting that the cellular *myo*-inositol level is the crucial factor for maintenance of normal nerve function [8,11].

In the present report we examine the transport of *myo*-inositol by bovine retinal capillary pericytes in culture. Metabolizable hexoses such as D-glucose and D-galactose, but not non-metabolizable hexoses, inhibited *myo*-inositol uptake noncompetitively. The inhibition of *myo*-inositol uptake by the metabolizable hexoses was significantly reversed by Sorbinil, an inhibitor of aldose reductase.

This work was presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, FL, May 10, 1985.

Materials and Methods

Cell cultures. Cultures and subcultures of bovine retinal capillary pericytes were prepared as previously described [2–6]. The purity of subcultured pericytes was accessed by phase-contrast microscopy, by the absence of indirect immunofluorescent staining for factor VIII antigen, and by the lack of angiotensin-converting enzyme activity. In collaboration with Dr. Lawrence E. Stramm, selected confluent primary and subcultured pericytes were fixed for electron microscopy in cacodylate-buffered 2.5% glutaraldehyde (pH 7.4), postfixed in 1% osmium tetraoxide, dehydrated, and embedded in Poly/Bed 812 media (Polysciences). Thin sections (silver interference) were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-9 S-2 electron microscope. Pericytes also were stained with nitrobenzoxadiazolylphallacidin, a fluorescent dye which specifically identifies actin filaments [16]. Subcultured pericytes from the 5th passage were replated in 24-well plates at an identical seeding density ($2.5 \cdot 10^4$ cells/cm^2) and used for transport studies 4 days after confluency, at a point at which the cells were in a stationary phase of the cell cycle [2].

Transport measurements. The time-course of *myo*-inositol uptake (zero-*trans*) was determined by incubating pericytes in 150 mM NaCl/2.0 mM $CaCl_2$/20 mM Hepes (pH 7.4) (buffer I) with 0.05 mM *myo*-[2-^{3}H]inositol (2.5 μCi/ml, 14 Ci/mmol) at 37°C in a water-bath shaker for 0–40 min. Uptake was terminated by rapid washing the cells (five times) in ice-cold buffer I containing 0.01 mM cytochalasin B (experimentally predetermined to inhibit *myo*-inositol uptake more than 90%). Pericyte accumulation of *myo*-[2-^{3}H]inositol was maximal by 15 min with no significant change between 15 and 40 min. A 20-min preincubation with unlabeled *myo*-inositol, therefore, was selected to characterize the kinetic parameters of *myo*-inositol transport under equilibrium-exchange conditions. The experiments were initiated by adding 1 ml of buffer I per well, and incubation was continued for 15 min. After removal of the buffer solution, the pericytes were preincubated with 0.5 ml/well of buffer I containing 0.005–40 mM unlabeled *myo*-inositol (adjusted to constant molarity with mannitol) for 20 min. At the end of the preincubation period, the preincubation solutions were replaced with the same solutions now containing *myo*-[2-^{3}H]inositol (2.5 μCi/ml, 14 Ci/mmol). The accumulation of *myo*-[2-^{3}H]inositol was monitored at 5–30-min intervals up to 90 min. Uptake was terminated by rapidly washing the cells (five times) with ice-cold buffer I containing 0.01 mM cytochalasin B. The cells were harvested and the radioactivity was determined in a scintillation spectrometer. To examine the effect of sodium ion concentration on *myo*-inositol uptake, the NaCl in buffer I was partially or totally replaced by LiCl (total NaCl and LiCl molarity 150 mM). The sodium-dependent *myo*-inositol uptake was calculated by subtracting the uptake at zero sodium concentration from that at higher sodium concentrations. In some instances, the NaCl of buffer I was replaced with choline chloride. To ascertain whether increasing lithium concentrations had a direct effect on *myo*-inositol uptake, the 150 mM NaCl of buffer I was replaced with 10 mM NaCl and 50–140 mM LiCl.

To study the effects of various compounds on *myo*-inositol or 3-*O*-methyl-D-glucose utpake by pericytes, cells were initially incubated with buffer I for 15 min, followed by a 20–59 min preincubation with buffer I containing either 0.05 mM *myo*-inositol or 5 mM 3-*O*-methyl-D-glucose and the

various compounds of interest. At the end of the preincubation periods, the medium was replaced by the same solutions now containing *myo*-[2-^{3}H]inositol (2.5 μCi/ml, 14 Ci/mmol) or 3-*O*-methyl-D-[1-^{3}H]glucose (10 μCi/ml, 2.1 Ci/mmol). The incubation was terminated either after 1 min for the determination of 3-*O*-methyl-D-glucose uptake [7], or after 20 min for the determination of *myo*-inositol uptake. In the experiments examining the effects of various hormones on *myo*-inositol uptake, the cells were preincubated with serum-free medium for 12–16 h in order to deplete exogenous hormones prior to the *myo*-inositol uptake studies.

To examine the efflux of *myo*-inositol from pericytes, after a 15-min incubation in buffer I, the cells were preincubated in buffer I containing either 0.1 mM phloretin, 0.05 mM *myo*-[U-^{14}C]inositol (1.25 μCi/ml, 333 mCi/mmol) and 0.63 μM [^{3}H]inulin (1 μCi/ml, 1.59 Ci/mmol), or 0.05 mM *myo*-[U-^{14}C]inositol and 0.63 μM [^{3}H]inulin, for 1 h. After removal of the labeling solutions and rapid washing with buffer I containing 0.05 mM unlabeled *myo*-inositol, either 0.1 mM phloretin and 0.05 mM unlabeled *myo*-inositol, or 0.05 mM unlabeled *myo*-inositol alone, was introduced (0.5 ml per well). Samples of the incubation solution (10 μl) were periodically removed for determination of radioactive *myo*-inositol by thin-layer chromatography (vide infra), or for measurement of the total radioactivity (*myo*-[U-^{14}C]inositol and [^{3}H]inulin). The residual radioactive inulin after washing was used to calculate the residual extracellular radioactive *myo*-inositol.

Determination of intracellular water space. The intracellular water space of cultrued retinal capillary pericytes was determined by the method of Kletzein et al. [17]. The cells were equilibrated with 1.25–10 mM 3-*O*-methyl[1-^{3}H]glucose by incubation for 30 min, and then washed (five times) with ice-cold buffer I containing 1 mM phloretin [7,13]. The data obtained with [^{3}H]inulin in a separate experiment were used to correct for the extracellular adherence of 3-*O*-methyl[1-^{3}H]glucose.

Assays. Radioactive *myo*-inositol samples (10 μl) were co-chromatographed with unlabeled *myo*-inositol (0.7%, w/v) in one-dimension on silica gel TLC plates (2 × 20 cm) in ethyl acetate/pyridine/water (120 : 50 : 40, v/v) and the chromatograms were developed with a silver nitrate reagent [18]. The R_F value of *myo*-inositol was obtained relative to D-glucose (R_g = 100). Cellular deoxyribonucleic acid (DNA) was determined by a method modified from Scott et al. [19], with authentic DNA as standard.

Statistical analyses were done by Student's two-tail *t*-test. Best-fit linear-regression lines were determined by the method of least-squares.

Materials. *myo*-[2-^{3}H]Inositol, *myo*-[U-^{14}C]inositol, 3-*O*-methyl-D-[1-^{3}H]glucose and [^{3}H]inulin were purchased from Amersham. Sorbinil was a gift from Dr. N.J. Hutson of Pfizer Inc. TLC plates were obtained from Whatman Chemical Inc. The remaining chemicals and reagents were purchased from Sigma Chemical Co. and were of the highest purity available.

Results

Pericyte cultures

The cultured pericytes were not reactive immunofluorescently for factor VIII antigen and lacked angiotensin-converting enzyme activity, both of which were demonstrated in cultured bovine retinal capillary endothelial cells [2]. Pericytes stained with nitrobenzoxadiazolylphallacidin contained many brightly fluorescent yellow filaments arranged in bundles or fans. By electron microscopy, the confluent pericytes tended to form a double-layer, and contained moderate numbers of microfilaments which usually were oriented in parallel bundles, rough endoplasmic reticulum, mitochondria and a Golgi apparatus. The pericytes produced an extracellular matrix, and glycosaminoglycans which differed significantly from those produced by cultured fibroblasts [20].

Kinetic analysis of myo-inositol uptake

The time-course for *myo*-[2-^{3}H]inositol uptake into pericytes under equilibrium-exchange conditions, and the sodium-dependent *myo*-inositol uptake, are shown in Fig. 1. Linearity was observed between 0 and 30 min of incubation in all curves ([Na^+] = 150 or 0 mM) with all *myo*-inositol concentrations tested (0.005–40 mM, all data not shown). Therefore, a 20-min incubation period was chosen to determine the sodium-dependent

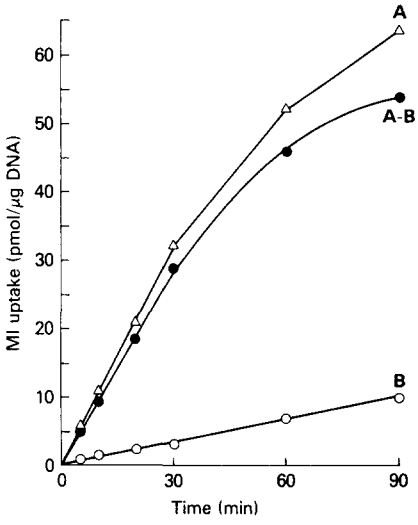

Fig. 1. Time-course of *myo*-inositol (MI) uptake by pericytes. Cells were sequentially preincubated with either buffer I, or sodium-free LiCl buffer I (15 min), followed by the same buffer containing 0.05 mM *myo*-inositol (20 min), and then incubated with buffer containing 0.05 mM *myo*-[2-^{3}H]inositol (2.5 μCi/ml) for the time indicated, at 37°C. Each point is the mean of five determinations. (A) (Δ) *myo*-inositol uptake when [Na^+] = 150 mM; (B) (○) *myo*-inositol uptake when [Na^+] = 0 mM; (A-B) (●) sodium-dependent *myo*-inositol uptake.

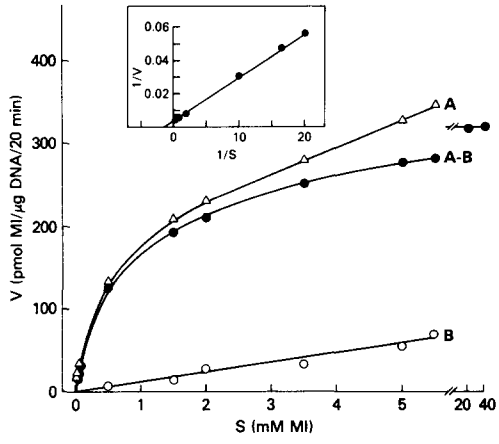

Fig. 2. Michaelis-Menten plot of sodium-dependent *myo*-inositol (MI) uptake by pericytes. (A) Total *myo*-inositol uptake (Δ), [Na^+] = 150 mM; (B) sodium-independent *myo*-inositol uptake (○), [Na^+] = 0 mM; (A-B) sodium-dependent *myo*-inositol uptake (●). Each point is the mean of five determinations. The inset presents a double-reciprocal Lineweaver-Burk plot of the sodium-dependent *myo*-inositol uptake data, and gave an apparent K_m of 0.83 mM.

initial velocities. A plot of the initial velocity of the *myo*-inositol uptake (at [Na^+] = 150 mM) against varying *myo*-inositol concentrations ex-

TABLE I

THE EFFECT OF ENERGY POISONS, PHLORIZIN, OUABAIN AND SODIUM ION CONCENTRATION ON THE UPTAKE OF 3-*O*-METHYL-D-GLUCOSE OR *myo*-INOSITOL

Results are means ± S.D. from five determinations. Together with LiCl, in all three cases, 10 mM NaCl was added.

Addition	3-*O*-Methyl-D-glucose (5 mM) uptake (nmol/μg DNA per min)	*myo*-Inositol (0.05 mM) uptake (pmol/μg DNA per 20 min)
None (control)	0.30 ± 0.04	18.22 ± 1.73
Potassium cyanide (1 mM)	0.32 ± 0.07	12.62 ± 1.26 *
2,4-Dinitrophenol (0.1 mM)	0.32 ± 0.05	13.35 ± 1.60 *
Phlorizin		
0.2 mM	0.29 ± 0.10	9.18 ± 0.94 *
1.0 mM	0.27 ± 0.15	5.69 ± 0.73 *
Ouabain		
$0.5 \cdot 10^{-5}$ M	0.31 ± 0.05	10.60 ± 1.15 *
$1.0 \cdot 10^{-5}$ M	0.30 ± 0.07	9.08 ± 1.07 *
$1.0 \cdot 10^{-4}$ M	0.29 ± 0.04	7.28 ± 1.19 *
LiCl		
140 mM	0.26 ± 0.08	2.63 ± 0.42 *,**
100 mM		2.56 ± 0.39 *,**
50 mM		2.50 ± 0.47 *,**

* Differed significantly from control, $P < 0.01$.

** No significant difference between results.

hibited a biphasic curve (Fig. 2). The rate of *myo*-inositol uptake in sodium-free medium increased linearly with increasing *myo*-inositol concentration. The *myo*-inositol uptake by pericytes consisted of two transport components, one sodium-independent and nonsaturable, and the other sodium-dependent (the *myo*-inositol uptake at 150 mM NaCl minus the uptake in sodium-free medium) and saturable at about 40 mM of *myo*-inositol (Fig. 2). Kinetic constants for the sodium-dependent component of the *myo*-inositol uptake were determined by the Lineweaver-Burk transformation of the Michaelis-Menten equation (Fig. 2, inset). The resulting curve gave an apparent K_m of 0.83 mM and V_{max} of 313 pmol *myo*-inositol/μg DNA per 20 min.

Varying the concentration of lithium in the medium, in the presence of a constant concentration of sodium, did not change the initial velocity of *myo*-inositol uptake (Table I). Replacement of sodium with choline produced the same sodium-dependent reduction of *myo*-inositol uptake as that obtained by replacement of sodium with lithium.

The requirement for external sodium in *myo*-inositol uptake at different concentrations of *myo*-inositol (0.005 or 0.05 mM) is further illustrated in Fig. 3. The initial velocity of *myo*-inositol uptake was reduced by 85% when NaCl (150 mM) was completely replaced by LiCl of equal molarity. After subtraction of the *myo*-inositol uptake in sodium-free medium, a plot of the data according to the Lineweaver-Burk equation (Fig. 3, inset) showed that the concentration of sodium required to produce one-half the maximal rate of uptake ($[Na^+]_{0.5}$) was independent of the *myo*-inositol concentration (22.3 mM for 0.005 mM *myo*-inositol, and 18.2 mM for 0.05 mM *myo*-inositol). When these data were replotted in Fig. 4 according to the Hill equation [20], the values of the slopes of the least-squares best fit linear-regression lines (n_H) were 1.1 for 0.05 mM *myo*-inositol and 1.0 for 0.005 mM *myo*-inositol. The values for $[Na^+]_{0.5}$ corresponding to a value of 1.0 on the y-axis of the Hill plots (Fig. 4) were the same as those derived from Fig. 3 (inset).

The kinetic parameters (K_m and V_{max}) at different concentrations of sodium were calculated according to the Lineweaver-Burk equation and are presented in Fig. 5. The apparent K_m for *myo*-inositol uptake was not dependent on the concentration of sodium ion. However, the V_{max} increased markedly with increasing sodium concentration (Fig. 5). When the *myo*-inositol uptake was determined in the presence of ouabain, a

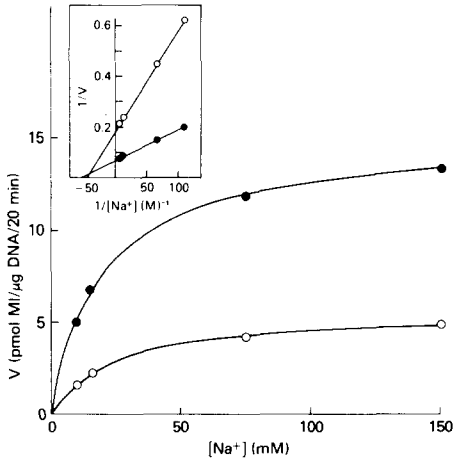

Fig. 3. Transport of *myo*-inositol (MI) as a function of external sodium ion concentration. Cells were preincubated with either 0.005 (○) or 0.05 mM (●) *myo*-inositol, with from zero to 150 mM sodium (NaCl replaced with LiCl), and then incubated with the same solution containing *myo*-[2-^{3}H]inositol (2.5 μCi/ml) for 20 min at 37°C. The uptake in the absence of sodium was subtracted from each experimental result. Each point is the mean of six determinations from two cell preparations. In the inset, the data are analyzed by a Lineweaver-Burk plot.

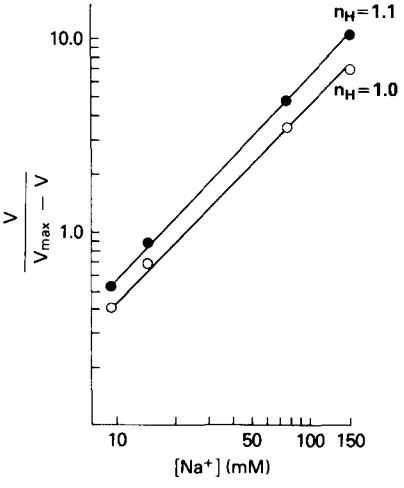

Fig. 4. The data of Fig. 3 replotted according to the Hill equation (0.05 mM *myo*-inositol (●); 0.005 mM *myo*-inositol (○)).

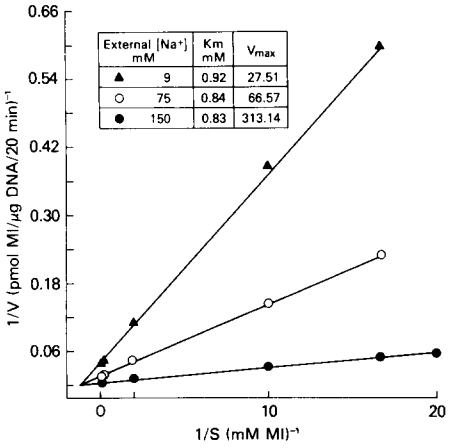

Fig. 5. Effect of external sodium concentration on the kinetic parameters of *myo*-inositol (MI) transport. Cells were preincubated with either buffer I (150 mM NaCl, ●), or LiCl-modified buffer I (9 mM NaCl, ▲; 75 mM NaCl, ○), containing 0.05 mM *myo*-inositol, and then incubated with the same buffer containing *myo*-[2-^{3}H]inositol (2.5 μCi/ml) for 20 min. Each point is the mean of nine determinations from triplicate experiments. The inset is a table of kinetic parameters determined from a linear-regression analysis of the Lineweaver-Burk plots presented in this figure.

potent inhibitor of sodium-potassium ATPase, a low concentration of ouabain ($0.5 \cdot 10^{-5}$ M) produced a 42% inhibition of *myo*-inositol uptake (Table I). The inhibitory effect on *myo*-inositol uptake was dependent on the ouabain concentration (Table I).

Effects of inhibitors of glucose transport and mitochondrial ATP synthesis, Ca^{2+} and Mn^{2+} on myo-inositol transport

Table II shows that 0.01 mM cytochalasin B inhibited the uptake of 0.05 mM *myo*-inositol at 37°C by 53%. The inhibition of *myo*-inositol efflux from pericytes by 0.1 mM phloretin is shown in Fig. 6. Phloretin (0.1 mM) which was either preincubated for 1 h, or added just prior to the initial measurement of extracellular *myo*-[U-^{14}C]inositol, suppressed the rate of efflux of *myo*-inositol as compared with the corresponding controls. Phlorizin showed a dose-dependent inhibition of the *myo*-inositol (0.05 mM) uptake (Table I). Mercuric chloride (1.0 mM) markedly suppressed *myo*-inositol uptake (Table II). Inhibitors of mitochondrial ATP synthesis, such as potassium cyanide (1 mM) and 2,4-dinitrophenol (0.1 mM),

TABLE II

THE EFFECT OF VARIOUS COMPOUNDS ON THE UPTAKE OF *myo*-INOSITOL

Results are means ± S.D. for five determinations.

Addition	*myo*-Inositol (0.05 mM) uptake (pmol/μg DNA per 20 min)
None (control)	18.22 ± 1.73
$HgCl_2$ (1.0 mM)	11.85 ± 1.42 *
$CaCl_2$ (0.2 mM)	18.62 ± 0.76
(0.8 mM)	19.02 ± 1.15
(2.0 mM)	17.59 ± 0.87
$MnCl_2$ (10 mM)	17.98 ± 1.70
(100 mM)	17.53 ± 2.14
Ethanol (1%, control)	15.12 ± 1.36
Cytochalasin B (0.01 mM) [a]	7.05 ± 0.53 *
NaOH (0.001 M, control)	17.80 ± 1.10
Sorbinil (0.1 mM) [b]	17.65 ± 0.98
Epinephrine (10 μM) [b]	16.98 ± 2.40
Angiotensin-II (0.1 μM) [b]	18.60 ± 1.03
Insulin (10 μg/ml) [b]	18.67 ± 0.96
Glucagon (0.1 μM) [b]	19.09 ± 1.82

[a] In 1% ethanol.
[b] In 0.001 M NaOH.
* Differed significantly from control, $P < 0.01$.

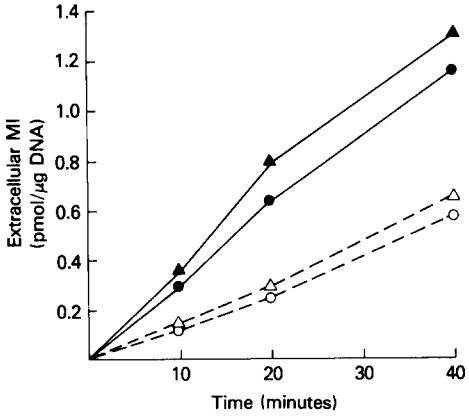

Fig. 6. The inhibition by phloretin of *myo*-inositol (MI) efflux from pericytes. Cells were preincubated with either 0.1 mM phloretin, 0.05 mM *myo*-[U-^{14}C]inositol and [^{3}H]inulin (●, ○) or with 0.05 mM *myo*-[U-^{14}C]inositol and [^{3}H]inulin (▲, △) at 37°C for 1 h. After removal of the preincubation solutions and rapid washing, either 0.1 mM phloretin and 0.05 mM unlabeled *myo*-inositol (– – –), or unlabeled 0.05 mM *myo*-inositol alone (———) was introduced. *myo*-[^{14}C]Inositol was determined by TLC. Each point is the mean of three determinations.

TABLE III

INHIBITORY EFFECT OF D-GLUCOSE ON *myo*-INOSITOL UPTAKE, AND REVERSAL BY SORBINIL

Results are means ± S.D. for five determinations.

D-Glucose (mM)	*myo*-Inositol (0.05 mM) uptake (pmol/μg DNA per 20 min)	D-Glucose effect (%)	*myo*-Inositol (0.05 mM) uptake with 0.1 mM Sorbinil (pmol/μg DNA per 20 min)	D-Glucose plus Sorbinil effect (%)
0	23.0 ± 4.5	100.0	22.9 ± 4.4	100.0
5	21.0 ± 1.8	91.3	22.3 ± 4.5	97.0
20	12.5 ± 1.6 *	54.3	18.8 ± 1.7 **	81.7
40	9.6 ± 1.2 *	41.7	14.6 ± 0.4 *,**	63.4
80	7.5 ± 0.5 *	32.6	10.4 ± 0.8 *,**	45.2

* Differed significantly from control (0 mM D-glucose), $P < 0.005$.

** Differed significantly from D-glucose effect without 0.1 mM Sorbinil, $P < 0.005$.

significantly inhibited 0.05 mM *myo*-inositol uptake by pericytes (Table I). In contrast, 3-*O*-methyl-D-glucose uptake by pericytes was not affected by the same concentrations of either potassium cyanide, 2,4-dinitrophenol, ouabain, or phlorizin (Table I). No significant effect of calcium or manganese ion concentration on *myo*-inositol uptake was observed (Table II).

Intracellular concentration of myo-inositol

The intracellular water space was determined to be 0.39 μl per μg of cellular DNA. At a low concentration of *myo*-inositol (5 μM) and 2 h incubation, the ratio of intracellular to extracellular concentration of *myo*-inositol was 17.8. When the *myo*-inositol concentration was increased to 50 μM, the ratio was much lower (4.8) than that obtained with 5 μM *myo*-inositol, but still was greater than unity.

Effects of hormones on myo-inositol transport

The effect of selected hormones on *myo*-inositol (0.05 mM) uptake is shown in Table II. Insulin, glucagon, epinephrine and angiotensin-II had no significant effect on *myo*-inositol uptake.

Effects of hexoses and an aldose reductase inhibitor on myo-inositol uptake

Cells were incubated with *myo*-inositol in the presence of D-glucose, its epimer, L-glucose, or its analogues, 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose. Since D-galactose, a member of the al-

TABLE IV

INHIBITORY EFFECT OF D-GALACTOSE ON *myo*-INOSITOL UPTAKE, AND REVERSAL BY SORBINIL

Results are means ± S.D. from five determinations.

D-Galactose (mM)	*myo*-Inositol (0.05 mM) uptake (pmol/μg DNA per 20 min)	D-Galactose effect (%)	*myo*-Inositol (0.05 mM) uptake with 0.1 mM Sorbinil (pmol/μg DNA per 20 min)	D-Galactose plus Sorbinil effect (%)
0	18.4 ± 1.0	100.0	18.1 ± 1.9	100.0
5	17.5 ± 3.9	95.1	18.7 ± 2.4	100.0
20	13.5 ± 2.0 *	73.4	17.9 ± 2.7 **	98.9
40	12.2 ± 2.5 *	66.3	16.2 ± 1.2 **	89.5
80	9.5 ± 1.1 *	51.6	12.0 ± 1.0 *,**	66.3

* Differed significantly from control (0 mM D-galactose), $P < 0.005$.

** Differed significantly from D-galactose effect without 0.1 mM Sorbinil, $P < 0.005$.

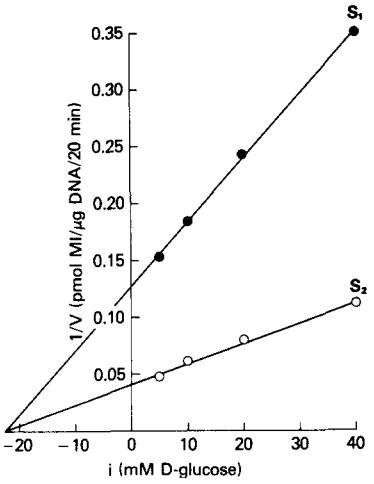

Fig. 7. Determination of the inhibition constant (K_i) by Dixon plot analysis for D-glucose inhibition of *myo*-inositol (MI) uptake by pericytes. Cells were incubated in either 0.01 mM (S_1, ●) or 0.05 mM (S_2, ○) *myo*-[2-^{3}H]inositol for 20 min. Each point is the mean of five determinations.

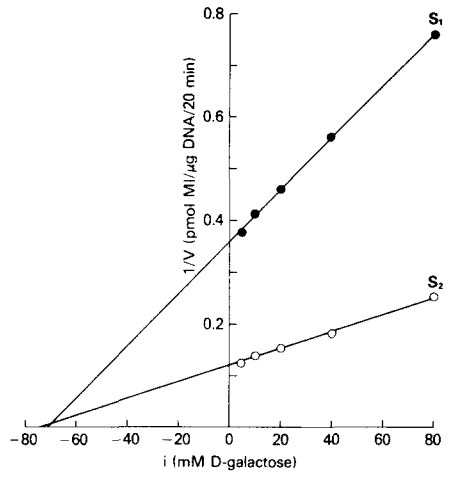

Fig. 8. Determination of the inhibition constant (K_i) by Dixon plot analysis for D-galactose inhibition of *myo*-inositol (MI) uptake by pericytes. Cells were incubated in either 0.005 mM (S_1, ●) or 0.02 mM (S_2, ○) *myo*-[2-^{3}H]inositol for 20 min. Each point is the mean of five determinations.

dohexose family, shares the sorbitol pathway with D-glucose, its effect on *myo*-inositol uptake also was examined. A physiological concentration (5 mM) of D-glucose did not significantly inhibit *myo*-inositol uptake as compared with that occurring in glucose-free media (Table III). However, a dose-dependent inhibition of *myo*-inositol uptake by D-glucose (above 5 mM) was demonstrated (Table III). The addition of Sorbinil, a potent aldose reductase inhibitor, significantly reduced the inhibition of *myo*-inositol uptake by D-glucose (higher than 5 mM) (Table III). Similarly, D-galactose (above 5 mM) inhibited *myo*-inositol uptake (Table IV). However, its inhibitory effect was less potent than that of D-glucose, and the reversal of this inhibitory effect by Sorbinil was more complete than that for D-glucose (Tables III and IV). In order to evaluate the nature of the

TABLE V

EFFECTS OF NON-METABOLIZABLE HEXOSES ON *myo*-INOSITOL UPTAKE

Results are means ± S.D. of six determinations.

Hexose (mM)	Sorbinil (mM)	*myo*-Inositol (0.05 mM) uptake (pmol/μg DNA per 20 min)		
		L-glucose	3-*O*-methyl-D-glucose	2-deoxy-D-glucose
0	0	18.0 ± 0.6	20.2 ± 1.4	19.6 ± 1.9
	0.1	18.1 ± 0.6	19.9 ± 1.2	19.4 ± 1.4
5	0	17.1 ± 1.0	19.9 ± 1.8	19.5 ± 2.7
	0.1	17.5 ± 1.1	19.1 ± 0.7	18.9 ± 1.2
20	0	16.8 ± 2.1	19.8 ± 1.4	18.2 ± 1.9
	0.1	16.6 ± 0.5	19.4 ± 1.0	18.7 ± 1.6
40	0	15.2 ± 1.7 *	19.2 ± 1.3	18.2 ± 1.0
	0.1	14.9 ± 1.6 *	19.4 ± 1.1	18.4 ± 2.0
80	0	13.8 ± 0.5 *	20.1 ± 1.9	15.9 ± 1.9 *
	0.1	14.2 ± 0.7 *	19.8 ± 0.5	16.0 ± 2.1 *

* Differed significantly from control (0 mM hexose), $P < 0.05$.

inhibition of *myo*-inositol uptake by D-glucose or D-galactose, different fixed concentrations of the substrate *myo*-inositol were incubated with varying concentrations of D-glucose (Fig. 7) or D-galactose (Fig. 8). The data were analyzed by Dixon plots [21,22]. In Fig. 7, the intersection of the Dixon plots for all values of substrate [S] on the inhibitor [i] axis at $[i] = -K_i$ indicated that the inhibition was non-competitive [21,22]. An inhibitor constant (K_i) for D-glucose of 22.7 mM was derived. D-galactose also functioned as a non-competitive inhibitor of *myo*-inositol uptake, with a K_i of 72.6 mM (Fig. 8). Double-reciprocal ($1/$initial velocity versus $1/$*myo*-inositol concentration) plots of the data of Fig. 7 or Fig. 8, at different fixed levels of either glucose or galactose, converged to a point to the left of the vertical ($1/v$) axis on the horizontal axis for each hexose inhibitor, again indicating non-competitive inhibition. The K_i for D-glucose inhibition was smaller than that of D-galactose, indicating that D-glucose was a more potent inhibitor of *myo*-inositol uptake than D-galactose (Tables III and IV). L-Glucose showed a small inhibition of *myo*-inositol (0.05 mM) uptake only at high concentrations (40 mM or greater) (Table V). The effect of 2-deoxy-D-glucose on the uptake of *myo*-inositol was similar to that obtained with L-glucose (Table V). 3-*O*-Methyl-D-glucose (up to 80 mM), a non-metabolized analogue of D-glucose, which is transported by the glucose carrier system in cultured pericytes [5], did not cause any change in *myo*-inositol (0.05 mM) uptake (Table V). The effects of L-glucose, 3-*O*-methyl-D-glucose, or 2-deoxy-D-glucose, on *myo*-inositol uptake were not significantly altered in the presence of 0.1 mM Sorbinil (Table V). Different concentrations of *myo*-inositol (0.05, 0.5, 10 mM) did not show any inhibitory effect on 3-*O*-methyl-D-glucose (0.05 mM) uptake by pericytes.

Discussion

The major transport system for *myo*-inositol uptake by cultured bovine retinal capillary pericytes was the active, saturable, carrier-mediated, sodium-dependent component which was inhibitable by ouabain. Lithium, a monovalent cation used to replace sodium in the medium in studies of the sodium dependence of *myo*-inositol uptake, may influence *myo*-inositol recycling by inhibiting inositol-1-phosphate phosphatase [23]. However, no lithium effect on *myo*-inositol uptake was seen when the lithium concentration was varied in the presence of a constant sodium concentration, or when choline replaced lithium. The *myo*-inositol uptake was relatively sensitive to phlorizin, a characteristic of a sodium-dependent transport [24]. *myo*-Inositol uptake was markedly decreased by potassium cyanide, an inhibitor of cytochrome *c* oxidase, or 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, indicating that the system was energy-dependent and required aerobic metabolism to support its energy demands.

The sodium-dependent *myo*-inositol transport appeared to be a co-transport process involving a ternary complex formed between the carrier, *myo*-inositol and sodium [25]. Within a physiological range of *myo*-inositol concentrations [26], the ternary complex had 1 : 1 stoichiometry between sodium and *myo*-inositol, since the slopes (n_H) of the linear-regression lines for the Hill equation plots were approximately unity [21,22]. The energy required for *myo*-inositol transport may be utilized to maintain the sodium gradient of the cellular membrane, and by energy transduction, to meet the demands for modification of the *myo*-inositol transporter [25]. The V_{max} for *myo*-inositol uptake was markedly changed with varying concentrations of sodium, but only minor changes in K_m were observed. *myo*-Inositol transport by pericytes, therefore, may fit a 'velocity type' co-transport model where binding of sodium to the transporter increased its mobility but not its affinity for *myo*-inositol [25,27].

We have reported elsewhere that D-glucose and its analogue, 3-*O*-methyl-D-glucose, were transported by pericytes by a sodium-independent, carrier mediated system which did not require metabolic energy (facilitated diffusion) [5]. Although the K_m values for the transport of *myo*-inositol and 3-*O*-methyl-D-glucose were similar (0.83 mM for *myo*-inositol; 1.53 mM for 3-*O*-methyl-D-glucose) [5], the K_m/K_i ratios for the inhibition of *myo*-inositol uptake by D-glucose (0.04), or for the inhibition of D-glucose uptake by *myo*-inositol [5], were not close to unity (both less than 0.05). This phenomenon argues against a sugar transport system sharing the same carrier [28]. Nevertheless,

high (above 5 mM) extracellular concentrations of D-glucose had a significant effect on *myo*-inositol transport by bovine retinal capillary pericytes. The inhibition of *myo*-inositol uptake by D-glucose was non-competitive.

The inhibitory effect of D-glucose on sodium-dependent *myo*-inositol uptake has been studied in the small intestine [29], in peripheral nerves [8–12,14] and in Schwann cells [15]. Caspary and Crane [29] reported that glucose was a non-competitive inhibitor of the transport of *myo*-inositol in the small intestine. Greene and Lattimer [10] reported that glucose was a competitive inhibitor of *myo*-inositol uptake in rabbit peripheral nerves, but presented conflicting kinetic data in which the modified Eadie-Hofstee plots indicated competitive inhibition whereas the Dixon plot was consistent with non-competitive inhibition [21,22].

In order to study further the nature of the non-competitive inhibition, both metabolizable and non-metabolizable hexoses were tested for their effect on *myo*-inositol uptake by pericytes. A high concentration of the non-metabolizable cyclic pyrans, L-glucose (20 mM) and 3-*O*-methyl-D-glucose (80 mM), and of the partially metabolizable cyclic pyran, 2-deoxy-D-glucose (40 mM), failed to inhibit *myo*-inositol transport. Therefore, it is unlikely that the inhibition of *myo*-inositol uptake by D-glucose is due simply to the structural similarity between the cyclic D-glucose and *myo*-inositol, or that a lack of inhibition by extracellular mannitol, sorbitol and galactitol [12,14,15] is due simply because these compounds are not cyclic. In contrast, a significant inhibition of *myo*-inositol uptake was produced by elevated concentrations (above 5 mM) of metabolizable D-glucose or D-galactose, suggesting that D-glucose or D-galactose metabolism was involved in the inhibitory effect on *myo*-inositol uptake (40 min incubation was sufficient for D-glucose or D-galactose metabolism to have taken place). D-Glucose and D-galactose both share the sorbitol pathway, and the inhibitory effect on *myo*-inositol uptake by each was significantly reversed by Sorbinil, an aldose reductase inhibitor. Sorbinil itself did not show any effect on *myo*-inositol uptake.

The inhibitory effect of D-glucose on *myo*-inositol transport may alter retinal microvascular phosphatidylinositol synthesis and turnover, and the formation of inositol trisphosphate and diacylglycerol [30,31]. Inositol trisphosphate has been shown to induce vascular calcium release and contraction [32], a potential function of retinal capillary pericytes in regulating vessel caliber [33]. The activity of transferases, such as CDP-diacylglycerol-inositol-phosphatidate-transferase which is responsible for the incorporation of *myo*-inositol in phosphatidylinositol synthesis, has a high K_m for *myo*-inositol, and may be just saturated at normal *myo*-inositol concentrations [34], and other enzymes of the cascade, also may be significantly influenced by a 40–50% reduction in microvascular *myo*-inositol uptake produced by the hyperglycemia of diabetes. The resulting altered cellular metabolism and pathophysiology may in turn play a major role in causing the more indolent structural pathologic changes of diabetic microvascular disease.

Acknowledgements

This work was supported by grants from the Juvenile Diabetes Foundation International, by NIH NEI Grant EY03984, by Fight for Sight, Inc., New York City, and by the Charles E. Goetz Teaching and Research Fund. The authors thank Dr. Lawrence E. Stramm for valuable discussions, and Virginia Mesibov and Bridgett Dent for the preparation of the manuscript.

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