Phosphate Transport by Jejunal Brush Border Membrane Vesicles of the Streptozocin-diabetic Rat

FAYEZ K. GHISHAN, STEPHEN BOROWITZ, AND ANDREW MULBERG

SUMMARY

We studied the effects of acute diabetes mellitus on jejunal transport of phosphate (^{32}P) by rat brush border membrane vesicles (BBMV) using a Millipore filtration technique. Diabetes was induced by an intravenous (i.v.) injection of 50 mg/kg of streptozocin (STZ). Control and diabetic rats were studied 4 days after the induction of diabetes. In both control and diabetic rats, the presence of a sodium gradient significantly enhanced the uptake of ^{32}P at 20 s and at 1, 2, 5, and 60 min as compared with potassium gradient conditions. Na^+-dependent ^{32}P uptake at 20 s and at 1 and 2 min was significantly greater in the diabetic BBMV compared with controls. Na^+-independent ^{32}P uptake in both diabetic and control BBMV was similar. To determine whether the enhancement of Na^+-dependent ^{32}P uptake in diabetic BBMV is due to an induction of Na^+/phosphate cotransporter activity, or a change in Na^+ permeability, two additional studies were conducted. Trans-stimulation studies nullifying all electrochemical gradients across the membranes were performed. In diabetic BBMV, ^{32}P uptake at all time points was significantly greater than corresponding values in controls, indicating an increase in the activity of Na^+/phosphate cotransporters. ^{22}Na uptake into BBMV at 30 s and at 1, 2, and 60 min was not different between diabetics and controls, indicating that Na^+ permeability is not altered in diabetes. Furthermore, kinetic studies using phosphate concentrations between 0.05 and 2.5 mM indicate a significant increase in V_{max} capacity of diabetic rats compared with controls without a change in K_m values. We conclude that the reason for the increased uptake of ^{32}P observed in 4-day diabetic BBMV is due to an increase in the activity of the Na^+/phosphate cotransporter. DIABETES 1985; 34:723–27.

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In the rat, chemically induced diabetes mellitus results in alterations of a variety of intestinal transport processes. An increase has been documented in the intestinal transport of hexoses,[1] amino acids,[2] and bile salts.[3] In contrast, the intestinal transport of calcium[4] and strontium has been shown to be depressed.[5] The transport of phosphate across the diabetic rat intestine has not been investigated. The described changes in intestinal transport are more pronounced in chronic diabetes mellitus; however, alterations have been shown to occur at 4–5 days after the induction of diabetes, when mucosal growth is similar between diabetics and controls.[6] The mechanism for these changes in intestinal transport remains largely unknown. Recently, the use of brush border membrane vesicles (BBMV) has allowed a more in-depth understanding of the transport processes across the intestinal mucosa.[7] Therefore, the present investigation was designed to examine the effects of diabetes mellitus on the uptake of phosphate into BBMV isolated from rat jejunum.

MATERIALS AND METHODS

Diabetes mellitus was induced in 250-g Sprague-Dawley rats with an i.v. injection of 50 mg/kg streptozocin (STZ) dissolved in citrate buffer. Control rats were sham injected with citrate buffer alone. Four days after injection, the presence of diabetes was verified by blood sugar concentrations > 300 mg/dl and the presence of glycosuria. Before killing, rats were fed standard chow and allowed free access to water. At the time of killing, the jejunum was removed from the ligament of Treitz until 25 cm distally, and washed with ice-cold 0.9 N saline. The intestine was then everted over a glass rod and the mucosa scraped free. The mucosal scrapings were homogenized using a Waring blender-type homogenizer at a maximum speed in 60 ml of 300 mM mannitol, 5 mM EGTA, and 12 mM Tris-HCl. The homogenate was treated with 3 ml of 1 M $MgCl_2$ and centrifuged in a Beckman J2-21 rotor (Beckman Instruments, Palo Alto, California) at 5000 rpm

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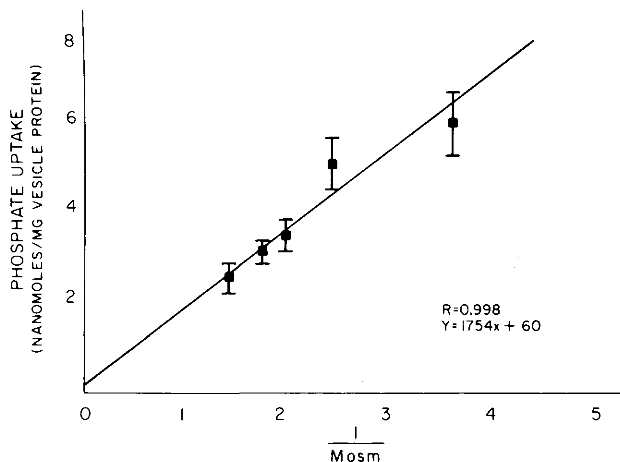

FIGURE 1. Jejunal BBMV were raised in 300 mM mannitol and 20 mM Hepes/Tris buffer (pH 7.4). Vesicle preparation was then incubated in a media containing 0.8 mM phosphate, tracer ^{32}P, and increasing concentrations of mannitol in an effort to increase the media osmolality. As seen, there is a linear relationship between media 1/osmolality and uptake. At infinite osmolality there is minimal binding (60 pmol/mg protein), indicating that phosphate uptake is into the intravesicular space. A similar relationship was noted in the diabetic rat BBMV.

(3000 × *g*) for 30 min. The resultant pellet was suspended in 60 ml of 60 mM mannitol, 5 mM EGTA, and 12 mM Tris-HCl at pH 7.4 and homogenized. The resultant homogenate was treated with 1.6 ml of 1 M $MgCl_2$ and then centrifuged for 15 min at 5000 rpm. The supernatant was centrifuged at 15,000 rpm (27,000 × *g*) for 30 min. The resulting pellet was resuspended in 40 ml of 280 mM mannitol and 20 mM Hepes/Tris pH 7.4 and centrifuged at 20,000 rpm (48,400 × *g*) for 30 min. The final pellet was resuspended with a syringe and 25-gauge needle in the desired volume of transport buffer containing 300 mM mannitol and 20 mM Hepes/Tris at a pH of 7.4.

Transport measurements. Uptake of phosphate was measured using a rapid filtration technique.[8] All experiments were performed at room temperature. Transport was initiated by adding 50 μl of the vesicle suspension to the desired incubation media containing potassium monophosphate-monobasic labeled with $KH_2\,^{32}PO_4$. The composition of the incubation media for each individual experiment is described in the figure legends of the RESULTS section. At the desired time intervals, 20-μl aliquots were removed and quickly diluted in 1 cc of ice-cold stop solution consisting of 100 mM mannitol, 100 mM choline chloride, 20 mM Hepes/Tris, and 50 mM magnesium chloride at pH 7.4. The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size, Sartorius Filters, Inc., Hayward, California) and kept under suction while being washed with 5 cc of ice-cold stop solution. The amount of radioactive phosphate remaining on the filter was determined in a liquid scintillation counter (Beckman Instruments) using Bray's solution (New England Nuclear, Boston, Massachusetts) as a liquid scintillant.

The protein content of the vesicle solution was determined by the method of Lowry et al.[9] using bovine serum albumin 1 mg/ml as a standard.

Most experiments described in this paper were performed three to six times, and each transport measurement was done in triplicate. Each experiment involved a minimum of six adult rats.

All results comparing experimental groups are expressed as means ± SEM. The significances of differences were calculated using the two-sided Student *t*-test.

Purity of the membrane preparation was assessed by the measurement of dissacharidases.[10] Leucine aminopeptidase was measured with the Boehringer kit 124869 (Boehringer, Indianapolis, Indiana). Na-K-ATPase activity was measured using the method of Wilson and Treavor.[11] In both control and diabetic BBMV, there was a 10–14-fold enrichment of the brush border markers in the final preparation as compared with the initial mucosal homogenate. There was no enrichment of Na-K-ATPase enzyme activity, indicating an absence of contamination with basolateral membranes.

RESULTS

Validation studies. Previous studies in our laboratory with D-glucose have demonstrated that intestinal BBMV are osmotically active. To determine whether phosphate enters the intravesicular space, vesicles were prepared in a Na^+-free solution and incubated in a Na^+-free buffer with mannitol concentrations varying from 60 to 560 mM. At 60 min, phosphate uptake by the vesicles was inversely proportional to the medium osmolality (Figure 1). Extrapolation to an infinite medium osmolality yields a phosphate uptake of 60 pmol/mg protein, indicating that phosphate uptake represents transport into the intravesicular space and not binding to the membrane surface.

Sodium gradient-dependent uptake of phosphate. To define the effects of diabetes mellitus on the sodium-dependent uptake of phosphate, jejunal BBMV from control and diabetic rats were prepared in a Na^+-free solution (300 mM mannitol and 20 mM Hepes/Tris buffer) and incubated in a Na^+-containing buffer at pH 7.4 (sodium outside the vesicle > inside the vesicle). In BBMV from the diabetic animals, phosphate

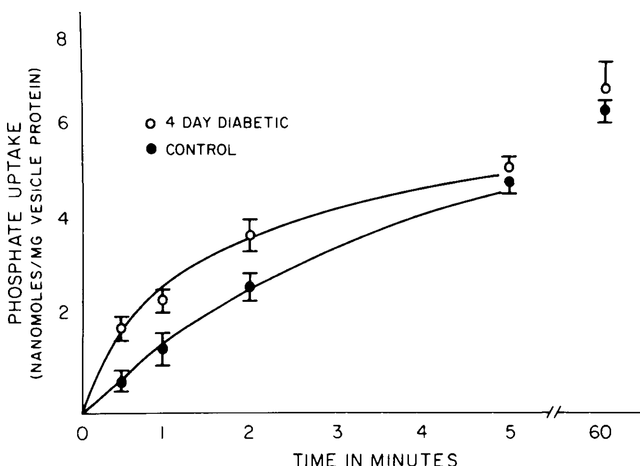

FIGURE 2. Jejunal BBMV from control and diabetic rats were raised in 300 mM mannitol and 20 mM Hepes/Tris buffer (pH 7.4) and then incubated in a media containing 100 mM NaCl (sodium outside the vesicle > inside the vesicle), 100 mM mannitol, 0.8 mM phosphate, and tracer ^{32}P. As seen in BBMV from the diabetic rats, phosphate uptake at 20 s and at 1 and 2 min was significantly greater than corresponding mean values in controls ($P < 0.05$).

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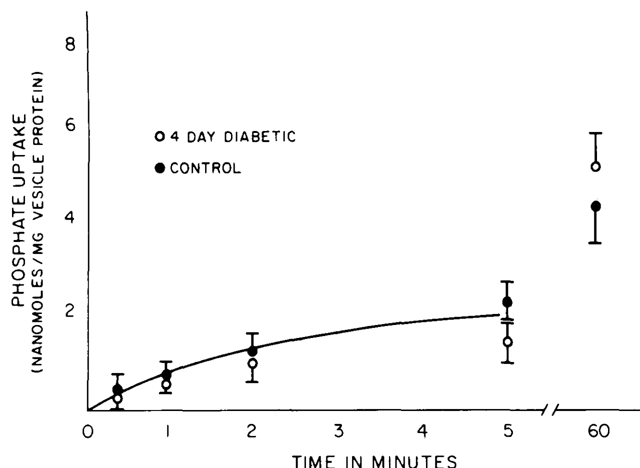

FIGURE 3. Jejunal BBMV from control and diabetic rats were raised in 300 mM mannitol and 20 mM Hepes/Tris buffer (pH 7.4) and then incubated in a media containing 100 mM KCl (K outside the vesicle > inside the vesicle), 100 mM mannitol, 0.8 mM phosphate, and tracer ^{32}P. Phosphate uptake at 20 s and at 1, 2, 5, and 60 min was similar in BBMV from both diabetic and control animals.

uptake at 20 s and at 1 and 2 min was significantly greater than corresponding values in controls ($P < 0.05$) (Figure 2).

Sodium-independent uptake of phosphate. To determine the effects of diabetes mellitus on the sodium-independent uptake of phosphate, jejunal BBMV from control and diabetic rats were prepared in a Na^+-free solution and incubated in a K^+-containing buffer at pH 7.4. Phosphate uptake at 20 s and at 1, 2, 5, and 60 min was similar in BBMV from both diabetic and control animals (Figure 3).

Effect of diabetes on trans-stimulation of phosphate uptake. To determine whether the observed difference in sodium-dependent phosphate uptake between the diabetic and control animals is secondary to a change in the activity of the sodium/phosphate cotransporter, trans-stimulation studies were performed. Vesicles were prepared in a Na^+-containing buffer with 0.8 mM potassium monophosphate and 6 μg/ml gramicidin at pH 7.4. The vesicles were incubated in pH-matched media containing Na^+, potassium monophosphate, and a tracer amount of radioactive phosphate. The sodium and phosphate concentrations in the preincubation and incubation media were identical. Gramicidin is an ionophorous antibiotic that increases the cation conductance of membranes.[12] It is used in this experiment to nullify all electrochemical gradients so that the only difference between the intra- and extravesicular space is a small gradient of radioactive phosphate. Any phosphate uptake taking place under these experimental conditions is due to the phosphate transporter. Under these experimental conditions, phosphate uptake in the diabetic BBMV at 20 s and at 1, 2, 5, and 60 min was significantly greater than the corresponding values in controls ($P < 0.01$, Figure 4). This suggests an increase in the activity or number of phosphate transporters present in the BBMV of the diabetic rats.

Effect of phosphate concentration on sodium-dependent uptake: kinetics of phosphate uptake. In an effort to define the kinetics of sodium-dependent phosphate transport, vesicles from control and diabetic rats were incubated in a Na^+-containing buffer with phosphate concentration varying from 0.05 to 2.5 mM. Uptake was measured at 10 s. Both in the presence and absence of Na^+, phosphate transported into the vesicles increased as the concentration of phosphate in the media was increased. A Lineweaver-Burk double-reciprocal plot of the active component of transport (sodium-dependent uptake minus sodium-independent uptake) demonstrates a V_{max} of 1.07 and 1.52 nmol phosphate/mg vesicle protein and a K_m of 0.096 and 0.12 mM phosphate for both control and diabetic rats, respectively. The difference between V_{max} in control and diabetic rats is significant, while K_m values are similar (Figure 5).

Effect of diabetes on ^{22}Na permeability. To determine whether the differences in phosphate uptake between diabetic and control rats are related to a change in Na^+ permeability, ^{22}Na uptake into BBMV from diabetic and control rats was determined. BBMV were preincubated in 300 mM mannitol and 20 mM Hepes/Tris buffer pH 7.4 for 60 min. The vesicles were then incubated at 37°C in a media containing 100 mM mannitol, 100 mM NaCl, 20 mM Hepes/Tris, and trace quantities of ^{22}Na at pH 7.4. Figure 6 depicts ^{22}Na uptake expressed as nmol/mg vesicle protein. At 30 s and at 1, 2, and 60 min, $^{22}Na^+$ uptake was not significantly different between diabetic and control rats.

DISCUSSION

Experimental diabetes mellitus is associated with multiple aberrations in growth and metabolism. The intestinal tract shows a 50% increase in mucosal growth at 12 days and a 100% increase at 32 days after the induction of diabetes. This increase in growth is associated with greater transport capacity for hexoses and amino acids.[1,2] However, 4 days after the induction of diabetes, there is an increase in the

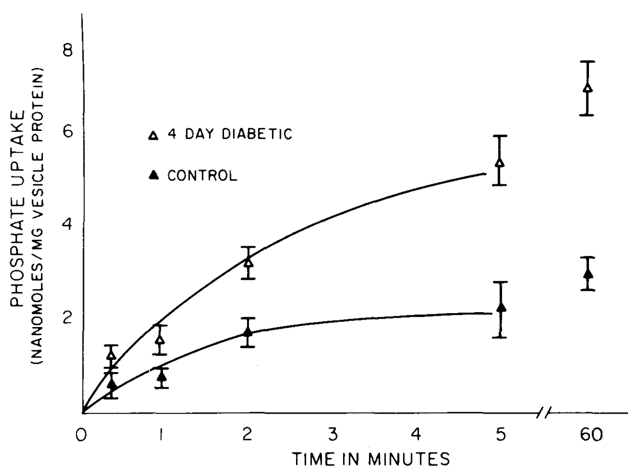

FIGURE 4. Jejunal BBMV from control and diabetic rats were prepared in a media containing 100 mM NaCl, 100 mM mannitol, 0.8 mM potassium monophosphate, and 6 μg/ml gramicidin at pH 7.4. The vesicles were then incubated in a pH-matched media containing 100 mM NaCl, 100 mM mannitol, 0.8 mM potassium monophosphate, and a tracer amount of ^{32}P. Under these experimental conditions, all gradients across the membranes were nullified except for the tracer amount of ^{32}P. Phosphate uptake, therefore, represents the Na-phosphate cotransporter activity. Phosphate uptake in diabetic rats at 20 s and at 1, 2, 5, and 60 min was significantly greater than the corresponding mean values in controls ($P < 0.01$).

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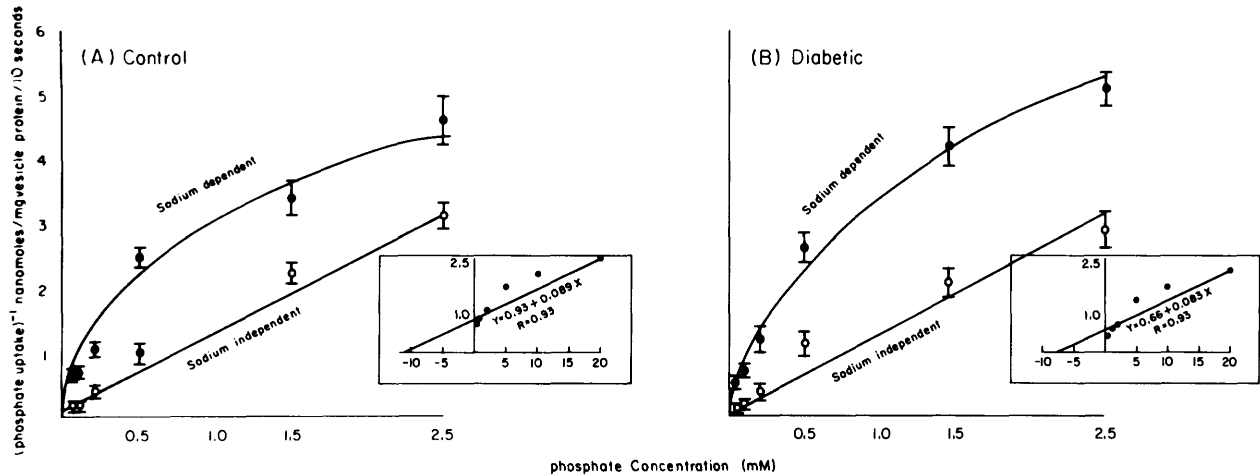

FIGURE 5. Lineweaver-Burk double-reciprocal plots were constructed for the active component of phosphate uptake (sodium-dependent minus sodium-independent uptakes) from control and diabetic animals: (●) sodium-dependent uptake, (○) sodium-independent uptake. For controls, the line y = 0.89x + 0.93 has a correlation coefficient of 0.93. K_m is 0.096 mM, and V_{max} is 1.07 nmol/mg of vesicle protein. For the diabetic animals, the line y = 0.083x + 0.66 has a correlation coefficient of 0.93. K_m is 0.12 mM, and V_{max} is 1.51 nmol/mg of vesicle protein.

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transport process of hexoses and amino acids, while mucosal growth is similar in both diabetics and controls. Thus, it appears that there is specific enhancement of these transport processes per se. This enhancement has not only been shown by in vivo and in vitro everted gut sac techniques,[1,2] but also by the use of BBMV.[13] Because both monactin and gramicidin result in a reduction in the active accumulation of D-glucose by BBMV in both diabetics and controls, it has been postulated that the increased uptake of glucose in diabetic animals is secondary to the ability of diabetic membranes to sustain a higher driving force for transport.[13]

The current studies extend previous observations on transport to include phosphate. The transport of phosphate across the small intestine of adult animals is an active process.[14] In rats, jejunal phosphate transport takes place against an electrochemical gradient and is inhibited by arsenate.[14] The uptake of phosphate at the brush border consists of two components: a saturable, electroneutral sodium-dependent component, and an unsaturable, sodium-independent component.[14] The energy driving the sodium-dependent entry of phosphate into the cell is provided by an extracellular-to-intracellular Na gradient. This gradient is maintained by the Na^+-K^+-ATPase at the basolateral membrane.[14] Our current results are in agreement with previous investigations that have demonstrated that the Na^+ gradient is the driving force for phosphate uptake across the brush border membrane. Moreover, membranes from diabetic rats show these same characteristics of phosphate transport, but with greater uptake as compared with controls. This increased uptake is not related to any increase in the ability of the diabetic membrane to sustain the Na^+ gradient, as ^{22}Na studies show no differences in the Na^+ permeability between controls and diabetics. The current studies, rather, demonstrate an increase in the activity of the Na^+/phosphate as a mechanism for the enhanced transport in the diabetic animals. This view has been recently supported by findings by Fedorak et al., in which not all Na-dependent absorptive processes are enhanced in diabetic rats.[15] For example, alanine and 3-O-methylglucose transport are enhanced in the ileum of diabetic rats; however, sulphate absorption, which is also a sodium-dependent process, is not enhanced in the same animals. If the mechanism for the enhanced transport in diabetes is the prolonged maintenance of the Na^+ gradient, all Na^+-dependent transport processes should be increased.

The short-circuit current changes produced by 3-O-methylglucose and alanine suggest that diabetes increases the transport V_{max} but has no effect on carrier affinity.[15] Our trans-stimulation studies and the kinetic data showing increase in the V_{max} but not the K_m suggest that the activity and/or the number of the phosphate is increased, supporting the observations of Fedorak et al. that the V_{max} capacity of transport is increased in diabetes mellitus. The mechanism for this increased activity of the carrier cannot be explained by the current studies. Gourley et al. have examined the composition and viscosity of the microvillous membrane from diabetic rats and found it to be similar to that of control rats.[16] There-

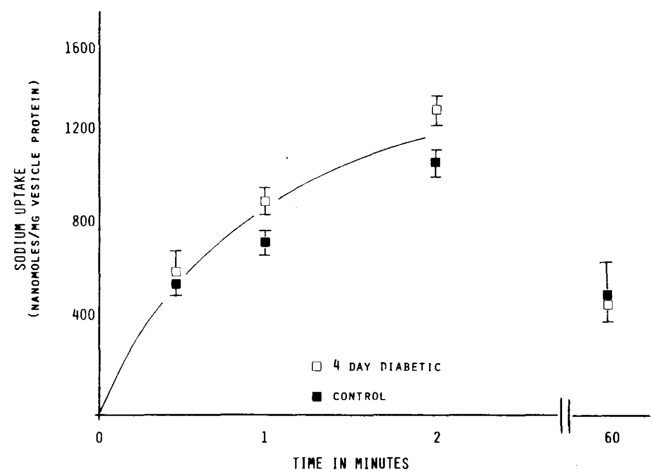

FIGURE 6. Jejunal BBMV from control and diabetic rats were raised in 300 mM mannitol and 20 mM Hepes/Tris buffer (pH 7.4). Membrane vesicles were then incubated in a media containing 100 mM NaCl, 100 mM mannitol, and ^{22}Na. Uptake of sodium at 30 s and at 1, 2, and 60 min was similar in control and diabetic BBMV.

fore, mechanisms other than changes in membrane composition should be sought to explain the increased activity of the Na^+-phosphate carriers of the diabetic rat intestine. Our results cannot be correlated with the decrease in $1,25(OH)_2$ vitamin D_3 noted in established diabetic rats, since we examined phosphate transport at 4 days after the induction of diabetes, while changes in $1,25(OH)_2$ vitamin D_3 have been described at 10 days after the induction of diabetes.[17] Longitudinal studies are essential to further elucidate this relationship.

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