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POLYAMINES STIMULATE D-GLUCOSE TRANSPORT IN ISOLATED RENAL BRUSH-BORDER MEMBRANE VESICLES

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Polyamines are natural constituents of most living organisms. However, their function in normal or pathological conditions is not fully understood. We have investigated in vitro effects of polyamines on characteristic properties of isolated renal brush-border membrane vesicles in order to determine whether polyamines have a regulatory role in membrane transport processes. The polyamines putrescine, spermidine and spermine were found to stimulate D-glucose uptake. Diffusional L-glucose uptake was not altered, indicating that the polyamines affected the active transport of D-glucose, rather than inducing nonspecific changes in membrane lipid properties. The amiloride-sensitive Na^+/H^+ exchange was slightly inhibited by polyamines while Mg^{2+}-ATPase activity was stimulated. The polyamine effects could not be explained solely by the polycationic properties of these agents, since polycationic polypeptides had an opposite effect. For example, lysozyme was found to inhibit D-glucose transport. Spermine was incorporated into the trichloroacetic acid-insoluble fraction of brush-border membrane proteins. Results indicated that this incorporation process consisted of at least two components: a Ca^{2+}-independent component and a Ca^{2+}-dependent component, possibly as a result of transglutaminase activity which was present in the isolated renal brush-border membranes. By using SDS-polyacrylamide gel electrophoresis in conjunction with fluorography, [^{3}H]spermine was shown to be incorporated into several brush-border membrane proteins, mainly the 57 kDa, 74 kDa, 100 kDa, a heavy molecular weight band (greater than 200 kDa) and a low molecular weight band (less than 10 kDa). Our results suggest that the polyamine effects on membrane function may be due to a covalent modification of membrane proteins, possibly via a transglutaminase-mediated incorporation of polyamines or to the crosslinking of membrane proteins.

Introduction

The aliphatic polyamines are ubiquitous in the mammalian body [1]. Micromolar concentrations are found in normal human tissues and blood plasma [2,3]. During normal embryonic growth, neoplastic growth, and in response to certain hormones and other agents that stimulate cell growth, a sharp increase in the key enzyme of polyamine biosynthesis, ornithine decarboxylase, is observed [3]. As a result, increased levels of polyamines have been found in rapidly growing animal tissues as well as in the blood plasma and urine of human subjects with a variety of neoplastic diseases [4–7]. In spite of their ubiquitous nature, the function of polyamines in normal or pathological conditions is not fully understood

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

and has been the subject of much controversy.

Under physiological conditions, polyamines are largely protonated and therefore exhibit a net positive charge [3]. It has been postulated that under certain conditions, polyamines may function as a replacement for essential cations such as Mg^{2+} [8]. Many of the functions of polyamines may relate to their serving as preferred cations for complexing with nucleic acids [3].

Several recent studies have suggested a possible regulatory role for polyamines in the process of endocytosis of certain polypeptide hormones. Evidence has been provided suggesting that transglutaminase may participate in the process of internalization, regulated by amines [9]. However, other studies have shown that the effect of amines was not obligatorily at the plasma membrane level and could instead be due to an effect on the intracellular processing of the ligand-receptor complexes [10]. Such a controversy is common to studies performed in vivo or those in isolated cells. In such systems, it is difficult to evaluate whether observed changes occur directly at the plasma membrane level, or whether they are indirect effects on the plasma membrane resulting from intracellular metabolic changes.

In the present study, we have used the brush-border plasma membrane vesicle of the renal proximal tubule as a model membrane to study, in vitro, the direct effect of polyamines on functions characteristic to this system. Since isolated brush-border membrane vesicles are an anucleate system lacking lysosomes and other intracellular organelles [11], many of the complications encountered in the interpretation of studies in whole cells are avoided. Studies in isolated brush-border membrane vesicles were therefore conducted to determine if polyamines play a regulatory role in processes occurring at the cell boundary.

Materials and Methods

Preparation of isolated brush-border membrane vesicles. Rabbit renal proximal tubule brush-border membrane vesicles were prepared as described previously [12,13]. After isolation, the membranes were washed twice in a medium containing 100 mM sucrose/100 mM KCl/2.5 mM $CaCl_2$/5 mM Tris-Hepes (pH 7.5). In experiments in which the effect of polyamines or other organic cations was measured, the membrane vesicles were incubated with a medium containing 100 mM sucrose/100 mM KCl/2.5 mM $CaCl_2$/5 mM Tris-Hepes (pH 7.5) and organic cations for 60 min at 37°C. All organic cation solutions were freshly prepared just prior to use. Following the incubation with the organic cations, membrane vesicles used to examine Na^+ uptake were washed twice in a medium containing 150 mM KCl/25 mM Mes/4.6 mM Tris (pH 5.5).

Transport measurements. Na^+ gradient-dependent D-[^{3}H]glucose uptake and ^{22}NaCl uptake by brush-border membrane vesicles was measured at 22°C by a Millipore filtration technique, using 0.45 μM filters ([13,14]. In D-glucose uptake studies, 20 μl of the membrane suspension (150–200 μg of protein) was preincubated for 1 min at 22°C and incubations were initiated by addition of 130 μl of uptake medium to give 100 mM sucrose, 100 mM NaCl, 50 μM D-[^{3}H]glucose (approx. 10^7 cpm), 5 mM Tris-Hepes (pH 7.5). Incubations were terminated by addition of an ice-cold solution containing 100 mM sucrose, 100 mM NaCl and 5 mM Tris-Hepes (pH 7.5). In Na^+ uptake studies, 20 μl of the membrane suspension (150–200 μg protein) was preincubated for 1 min at 22°C and incubations were initiated by addition of 130 μl uptake medium to give 144 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, 1 mM ^{22}NaCl (approx. 10^7 cpm) (pH 7.5). Incubations were terminated by addition of ice-cold 150 mM KCl/16 mM Hepes/10 mM Tris (pH 7.5) and rapid filtration on 0.45 μm filters. All incubations were carried out with fresh membranes. Each experiment was repeated at least three times with different membrane preparations. Results from representative experimets are given. Error bars represent standard deviations of replicate samples in the indicated experiment.

Assays. Protein was determined according to Lowry et al. [15], using bovine serum albumin as the reference protein. The ATPase assay was done using the method previously described by Yoda and Hokin [16]. All assays were carried out in triplicate.

Transglutaminase activity was measured essentially by the analytical method of Lorand et al.

[17]. Membranes used for this assay were isolated by $MnCl_2$ precipitation [14]. Membrane vesicles were loaded and dispersed in a solution containing 20 mM dithiothreitol, 53 mM Tris-HCl (pH 7.5) and either 1 mM EGTA or 10 mM $CaCl_2$. The assay was started by adding 50 μM [^{3}H]spermine. After incubation for various times at 37 °C, 7-μl aliquots were removed from the reaction mixture and spotted onto Whatman 3 MM filter papers (1 cm^2). The filters were immediately immersed in 10% trichloroacetic acid. In order to estimate nonspecific binding of spermine, the reaction in some of the samples was terminated immediately after the addition of spermine to the membranes. Accordingly, 7-μl aliquots were spotted on the filters and immediately immersed in 10% trichloroacetic acid. The labeling obtained on these filters was considered to be due to nonspecific binding and was subtracted from the values obtained after incubation for longer times. Processing and isotope counting were carried out as described [17]. Results are given with standard deviations of replicate samples in a representative experiment.

SDS-gel electrophoresis of renal brush-border membrane proteins. A sample of the membrane vesicle suspension was boiled in 1% SDS for 1 min. The sample was then electrophoresed on SDS-polyacrylamide gradient (linear 5–15%) gels with the buffers described by Laemmli [18]. The gels were stained with Coomassie blue according to Weber and Osborne [19] and subjected to fluorography utilizing en^{3}Hance (New England Nuclear) as recommended by the manufacturer. The molecular weight of specific bands was determined using the following molecular weight markers (Sigma): lysozyme (14.3 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa).

Materials. [^{3}H]Spermine, D-[^{3}H]glucose, L-[1-^{14}C]glucose and ^{22}NaCl were purchased from Amersham. Polyamines and lysozyme were purchased from Sigma Chemical Co. Amiloride-HCl was obtained from Merck.

Results

Effect of polyamines on D-glucose uptake

The general properties of the glucose uptake system in the rabbit renal brush-border membrane vesicles used in the present study resembled the uptake patterns reported in earlier studies [13,14]. In the presence of 50 μM putrescine, spermidine or spermine, the initial uptake rate was significantly stimulated (Fig. 1) at all times until equilibrium had been established, indicating that the polyamines did not alter the intravesicular volume. Additional evidence showing that polyamines affected the active transport of D-glucose rather than inducing nonspecific changes in membrane properties was obtained by testing the effects of spermine on L-glucose uptake. In Fig. 2, no significant effect of spermine on the diffusional L-glucose uptake could be shown, whereas D-glucose uptake increased significantly.

In contrast to the polyamines, the polypeptide lysozyme, a polycation at pH 7.5, had a dose-dependent inhibitory effect on D-glucose transport (unpublished data).

Effect of polyamines on Na^+ uptake

Na^+ reabsorption is an important function of the brush-border membrane in the renal proximal tubule, since it is coupled to the transport of water, electrolytes and certain organic compounds.

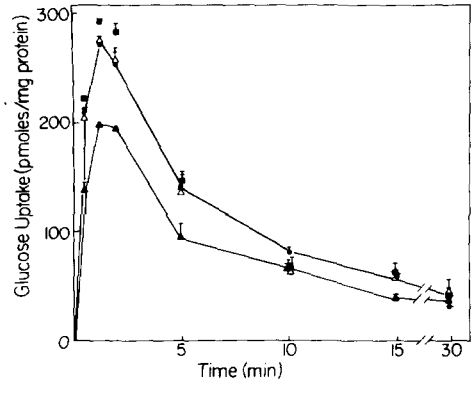

Fig. 1. Effect of polyamines on D-glucose uptake by renal brush-border membrane vesicles. Vesicles were first preloaded with 100 mM sucrose/100 mM KCL/2.5 mM $CaCl_2$/5 mM Tris-Hepes (pH 7.5) and then incubated with 50 μM polyamines (37 °C; 60 min). Following incubation, Na^+ gradient-dependent D-glucose transport was measured. The transport medium contained 100 mM sucrose/100 mM NaCl/50 μM D[^{3}H]glucose (approx. 10^7 cpm)/5 mM Tris-Hepes (pH 7.5). ▲, Control membranes were incubated under identical conditions with no polyamines added; ●, spermine; △, spermidine; ■, putrescine.

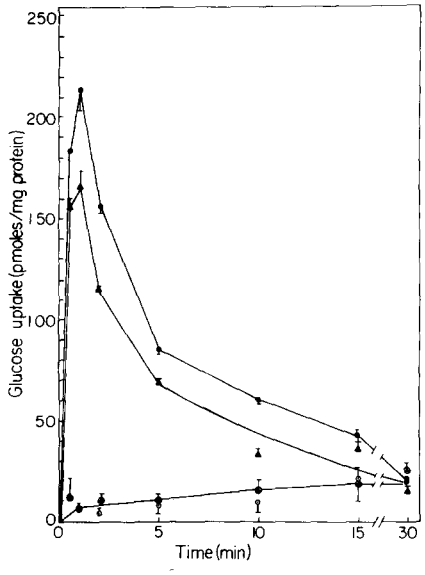

Fig. 2. Effect of spermine on D-glucose and L-glucose uptake by renal brush-border membrane vesicles. Preloading conditions and incubation with (●,⊙) or without (▲,◬) 50 μM spermine were as described in Fig. 1. The transport medium contained 100 mM sucrose, 100 mM NaCl, 5 mM Tris-Hepes (pH 7.5) and either 50 μM D[^{3}H]glucose (▲,●) or L[^{3}H]glucose (◬,⊙) (approx. 10^7 cpm).

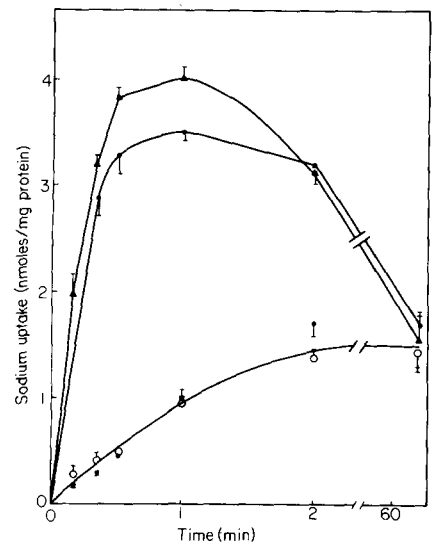

Fig. 3. Effect of spermine on Na^+ uptake. Preloading conditions and incubation with (●,⊙) or without (▲,○) 50 μM spermine were as described in Fig. 1. After incubation, membranes were loaded with 150 mM KCl, 2.5 mM Mes, 4.6 mM Tris (pH 5.5). After loading, Na^+ uptake was measured in the following medium: 144 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes (pH 7.5), 1 mM ^{22}NaCl (approx. 10^7 cpm) with (○,⊙) or without (▲,●) 1 mM amiloride.

Any change in Na^+ transport would be expected to affect other reabsorption processes as well. We have studied the effect of spermine on one of the main mechanisms of Na^+ transport, the amiloride-sensitive Na^+-H^+ antiport. Spermine appeared to cause a small, but significant, decrease in Na^+ uptake via the amiloride-sensitive Na^+-H^+ antiport mechanism. However, no significant effect on the amiloride-insensitive Na^+ uptake (mainly diffusional pathways) could be demonstrated under the conditions of this transport assay ([Na^+] = 1 mM]) (Fig. 3).

Effect of spermine on Mg^{2+}-ATPase activity

Spermine was found to stimulate Mg^{2+}-ATPase activity in the renal brush-border membrane with a maximal increase of about 30% over basal activity occurring at a spermine concentration of 50 μM (Table I). Interestingly, in the absence of magnesium, spermine was capable of sustaining about 10% of the total ATPase activity observed in the presence of magnesium (Table I). In the absence of both magnesium and spermine, the initial rate of ATPase activity was barely detectable (0.3 ± 0.2 nmol ATP hydrolyzed/mg protein per min). Addition of spermine resulted in a dose-dependent increase in ATPase activity (maximal activity: 17.6 ± 7.8 nmol ATP hydrolyzed/mg protein per min).

The nature of the interaction of polyamines with the renal brush-border membrane

Several possible mechanisms by which polyamines may affect membrane function were considered. The possibility that the observed polyamine effects were due to nonspecific binding to the membrane did not seem to be likely in the ionic environment in which the experiments were performed ([spermine] = 50 μM, [NaCl] = 100 mM, [$CaCl_2$] = 2.5 mM). Modification of renal brush-border membrane properties as a result of Ca^{2+}-stimulated covalent crosslinking of membrane proteins, possibly via a transglutaminase activity seemed to be an attractive possibility. Transglutaminases are a family of calcium-dependent enzymes that crosslink proteins by catalyzing the formation of γ-glutamyl-ε-lysine bonds. In the pre-

TABLE I

SPERMINE STIMULATION OF THE INITIAL RATE (5 min) OF ATPase ACTIVITY IN THE PRESENCE OR ABSENCE OF MAGNESIUM

Membrane vesicles were loaded with 200 mM sucrose, 50 mM Tris-HCl (pH 7.5) and incubated for 60 min at 37 °C with various concentrations of spermine. Following incubation, ATPase activity was determined in a reaction mixture containing 0.5 mM ATP, 200 mM sucrose, 50 mM Tris-HCl (pH 7.5) and various concentrations of spermine, with or without 0.5 mM $MgCl_2$. The experiment was repeated three times. Results from a representative experiment are given with the standard deviation of replicate samples.

Spermine (μm)	ATPase activity [a] (nmol ATP/ mg protein per min)	Mg^{2+}-ATPase activity [b]	
		nmol ATP/ mg protein per min)	% stimulation
0	0.3 ± 0.2	139.8 ± 4.0	
10	7.6 ± 0.1	159.9 ± 0.8	14.4
50	12.3 ± 0.8	185.7 ± 6.5	32.8
250	17.6 ± 0.8	163.1 ± 2.4	16.7

[a] No magnesium present; [ATP] = 0.5 mM.
[b] [ATP] = [Mg^{2+}] = 0.5 mM.

sent study, we attempted to measure transglutaminase activity in the brush-border membrane vesicles by assaying the incorporation of [^{3}H]spermine into the trichloroacetic acid-insoluble fraction of membrane proteins in the presence or absence of Ca^{2+}. The results (Fig. 4) demonstrated the presence of a Ca^{2+}-dependent incorporation of spermine into the trichloroacetic acid-insoluble fraction of membrane proteins.

To determine if spermine was being incorporated into specific membrane protein(s), membrane vesicles were incubated for 4 h in a reaction mixture containing 50 μM [^{3}H]spermine, 53 mM Tris-HCl (pH 7.5), 20 mM dithiothreitol and 10 mM $CaCl_2$. Following incubation, the membrane proteins were separated by SDS gel electrophoresis. The gel was stained for total protein and subjected to fluorography as described in Materials and Methods to identify proteins which contain [^{3}H]spermine. The staining pattern observed with Coomassie blue is given in Fig. 5A. The pattern of proteins was found to be similar to that in intestinal brush-border membranes [35]. In Fig. 5B is shown the autoradiograph of the gel in Fig. 5A. Spermine was incorporated into several groups of membrane proteins. A highly labeled fraction remained at the origin of the gel and may contain [^{3}H]spermine-labeled high molecular weight protein (Fig. 5B). A very marked spermine binding was also observed in a low molecular weight fraction of protein (6–10 kDa) (Fig. 5A and B).

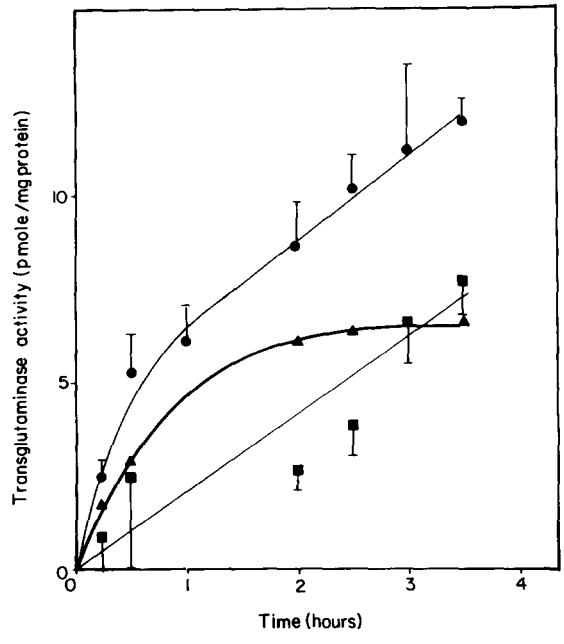

Fig. 4. Ca^{2+}-dependent incorporation of spermine into the trichloroacetic acid-insoluble fraction of brush-border membranes (transglutaminase activity). Membrane vesicles were loaded and dispersed in a solution containing 20 mM dithiothreitol, 53 mM Tris-HCl (pH 7.5) and either 1 mM EGTA (■) or 10 mM $CaCl_2$ (●). Transglutaminase activity was assayed in a solution containing 20 mM dithiothreitol, 53 mM Tris-HCl (pH 7.5), 50 μM [^{3}H]spermine (approx. 10^7 cpm) and either 1 mM EGTA (■) or 10 mM $CaCl_2$ (●). Calcium-dependent spermine incorporation (▲) was obtained by subtracting the values of the incorporation in the absence of Ca^{2+} (1 mM EGTA, ■) from the values of the incorporation in the presence of 10 mM $CaCl_2$ (●).

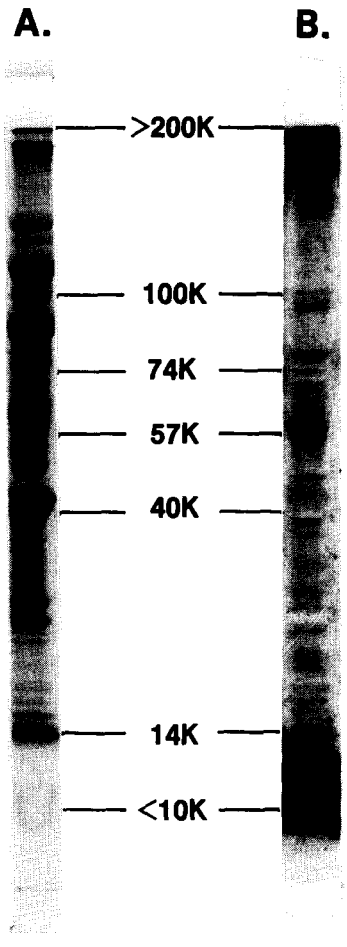

Fig. 5. SDS-gel electrophoresis of proteins in renal brush-border membrane vesicles. SDS-polyacrylamide gel electrophoresis was conducted as described in Materials and Methods. (A) Coomassie blue staining of renal brush-border membrane proteins, 4 h after incubation in a reaction mixture containing 50 μM [^{3}H]spermine, 53 mM Tris-HCl (pH 7.5), 20 mM dithiothreitol and 10 mM $CaCl_2$. (B) Autoradiograph of the gel in (A).

Discussion

In the present study, we have shown that polyamines stimulate D-glucose transport and ATPase activity in isolated renal brush-border membrane vesicles. The effect on D-glucose transport may be due to a direct effect of polyamines on the renal brush-border glucose carrier or it may be the result of an indirect effect on D-glucose transport as a result of inhibition of sodium uptake mechanisms. In the isolated membrane vesicle system, such inhibition prolongs the maintenance of the Na^+-gradient, on which the active D-glucose transport depends, thus stimulating the latter [14]. We have demonstrated a slight inhibition of the amiloride-sensitive Na^+/H^+ exchange by spermine. It is possible that under the conditions in which the glucose transport is measured ([NaCl] = 100 mM), other Na^+ transport mechanisms, such as the fast Na^+ exchange via Na^+ channels [24] are also inhibited by polyamines.

In earlier studies, it was shown that polyamines stimulated D-glucose transport in fat cells [20,21]. It was proposed that this effect resulted from the formation of H_2O_2 which had been shown to stimulate glucose transport in fat cells [22]. The enzyme diamine oxidase, which can use putrescine as a substrate to produce H_2O_2 is present in the hog kidney [3,23]. Therefore, the putrescine effects observed in our studies could have been due to the production of H_2O_2 via oxidation of putrescine by diamine oxidase. One mole of H_2O_2 would be expected to be produced per mole of putrescine oxidized [23]. However, since the addition of 50 μM H_2O_2 was found to have no significant effect of D-glucose transport in the isolated brush-border membrane (unpublished data), it is unlikely that the stimulatory effect of putrescine in this system was mediated by H_2O_2. In addition, diamine oxidase activity does not explain the spermine and spermidine effects, since these polyamines are not substrates for this enzyme [23].

An effect of polyamines in mediating the testosterone-induced stimulation of renal cortical membrane transport in mouse kidney has been suggested in a recent study in the cortex [25]. It was proposed that the polyamines served as intracellular signals to enhance Ca^{2+} influx across the plasma membrane and Ca^{2+} efflux from mitochondria and other organelles. The mechanism of this effect was suggested to be a cation-exchange reaction, whereby the polyamines bind to anionic sites in the membranes and competitively displace Ca^{2+} as free cytosolic Ca^{2+} [25]. Our study in membrane vesicles isolated from the renal cortex suggest that the polycationic nature of polyamines cannot fully account for their effects on transport. Under physiological conditions (approx. pH 7.4), all of the primary and secondary amino groups of putrescine, spermidine and spermine are

almost totally protonated, so that they exhibit net positive charges of approx. 2, 3, and 4, respectively. In spite of the different charge, the extent to which 50 μM putrescine, spermidine or spermine affected D-glucose transport was similar (Fig. 1). The fact that other polycationic compounds, such as lysozyme, had an opposite effect appears to indicate that the polyamines interact with the membrane in a more specific way. These results indicate that the effect of polyamines on D-glucose transport in the renal brush-border membrane vesicles cannot be explained by the polycationic nature of polyamines or by the production of H_2O_2.

Our results showed a direct stimulatory effect of spermine on renal brush-border Mg^{2+}-ATPase activity, and a limited but significant dose-dependent ability of spermine to replace the Mg^{2+} needed for ATPase activity. The activation of ATPase by magnesium is thought to occur primarily as a result of the magnesium reacting with ATP, reducing the high negative charge of the substrate by formation of MgATP which then reacts with the enzyme [36]. It is possible that spermine reacts with ATP in a similar way. However, our results indicate that even if such a complex, spermine-ATP, is formed, it is probably not the mechanism for the spermine effect on ATPase activity, since the spermine effect is maximal at a spermine/ATP ratio (0.05 mM:0.5 mM) which is much lower than 1:1 (Table I). Alternatively, spermine may have a role in the stabilization of the three-dimensional folding of the renal brush-border ATPase, analogous to similar effects observed in other systems [33,34].

The fact that polyamines stimulated two apparently unrelated functions, glucose transport and ATPase activity, indicates that their effect could be the result of a more general modification of membrane properties. The possibility that polyamines interact with and modify the properties of membrane lipids does not appear likely, since spermine was found to have no effect on the diffusional L-glucose transport (Fig. 2). A similar conclusion was reached in a study in erythrocytes, in which polyamines were shown to inhibit the lateral mobility of erythrocyte membrane proteins [26]. The effect was not due to changes in the membrane lipid state as determined by microviscosity experiments, but was attributed to restriction of the lateral mobility of membrane proteins as result of the interaction of spermine with the cytoskeletal proteins spectrin, actin and component 4.1 [29]. Earlier studies in erythrocytes [17] had also indicated the possibility that such an interaction existed and suggested further that an intrinsic membrane enzyme, transglutaminase, mediates protein crosslinking of the erythrocyte membrane whenever there is an increase in intracellular Ca^{2+} concentration [17].

Transglutaminases are a family of calcium-dependent enzymes that crosslink proteins by catalyzing the formation of γ-glutamyl-ϵ-lysine bonds. Transglutaminases act specifically on peptide-bound glutamine residues, and primary amino groups in a large group of compounds may serve as acceptor substrates [27]. Polyamines have been shown to be involved in post-translational modification of both intracellular [28–30] and membrane proteins [9,17,31]. Moreover, transglutaminase activity has been shown to be associated with a membrane fraction separated from kidney homogenates [32]. Using the standard assay procedure for transglutaminase [17], we were able to demonstrate a Ca^{2+}-dependent incorporation of spermine into the renal brush-border membrane proteins (Fig. 4). As in erythrocytes [17,26], this may be, in part, spermine incorporated into cytoskeletal proteins inducing their crosslinking into what appears to be a high molecular weight protein band (greater than 200 kDa). (Fig. 5). However, a significant Ca^{2+}-independent incorporation occurred as well (Fig. 4). Therefore, our results in isolated renal membrane vesicles are consistent with similar findings in the intact kidney system [25], and suggest that a cation-exchange reaction occurs as a result of a fast spermine-membrane protein interaction. This interaction is probably not Ca^{2+}-dependent, since Ca^{2+} is not needed for it to occur, but as a result of this interaction, Ca^{2+} bound to membrane proteins may be released. This could result in an increase of the intravesicular Ca^{2+} concentration activating Ca^{2+}-dependent enzymes, one of which may be transglutaminase. The Ca^{2+}-dependent crosslinking of membrane proteins occurring as a result of this activity may account for the modification of the renal membrane transport properties which are observed and

is consistent with the hypothesis that polyamines control renal membrane permeability via an interaction with the membrane proteins.

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