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Putrescine transport in human platelets

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Putrescine transport has been studied in human platelets. The uptake of putrescine is saturable and appears to be an energy-dependent process, since it is inhibited by the uncoupler 2,4-dinitrophenol and low temperature. The evidence presented suggests that the uptake process is complex and may be dependent upon pH gradient, membrane potential, and other unidentified factors. Putrescine transport is not inhibited by amino acids and is only slightly inhibited by spermidine and spermine. A membrane protein involved in putrescine transport has been identified and partially purified. Differential labeling with *N*-ethylmaleimide identified proteins with apparent molecular weights of 65000 and 23000 as determined by SDS-polyacrylamide gel electrophoresis. Column chromatographic purification on a putrescine affinity column revealed a M_r 55000 protein which copurified with the M_r 65000 protein. Additional evidence supporting the involvement of these proteins in putrescine transport was seen in putrescine protection against *N*-ethylmaleimide inhibition of putrescine uptake. Putrescine uptake may occur via the serotonin transport system, since imipramine inhibits transport and because of the similarities in the molecular weights of the proteins implicated in transport.

Introduction

Polyamines are ubiquitous organic cations which are present in significant amounts in all cells. While the physiological function of polyamines is still not understood, many biological effects have been ascribed to these compounds [1]. Some of the effects can be attributed to the polybasic nature of the polyamines. However, a large number of results indicate that the polyamines do not simply act by binding to negatively charged molecules.

We have studied putrescine transport in human platelets. Platelets are anucleate blood cells which circulate in blood whose aggregation plays an important role in hemostasis [3]. Polyamines have been shown to inhibit platelet aggregation [4–6]. The putrescine concentration found in whole blood is approx. 0.5 μM, with the majority of the polyamines in the blood being found within the various formed elements [2]. In disease states, such as cancer, cystic fibrosis and leukemia, the concentration of the polyamines in the blood can increase as much as 10-fold over normal levels; these increases may have important effects on platelet aggregation in vivo.

The basic characteristics of putrescine transport have been described for various cell types [1,7–11]. However, molecular mechanisms of polyamine transport have not been studied. The transport of putrescine into cells appears to be required for polyamine action [12,13]. This paper describes

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Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, FCCP carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SDS, sodium dodecyl sulfate.

studies of the energetics of putrescine transport in platelets and the identification of proteins which appear to be involved in the uptake process.

Materials and Methods

[^{3}H]Putrescine (0.24 Ci/mmol) was purchased from Amersham. *N*-[^{14}C]Ethylmaleimide (42.9 mCi/mmol) was purchased from New England Nuclear. *N*-Ethylmaleimide, deoxyribonuclease I, actin and valinomycin were purchased from Sigma. Activated CH-Sepharose 4B was purchased from Pharmacia Fine Chemicals. All other reagents were of the best available analytical grades.

Isolation of platelets. Whole blood anticoagulated with 3.8% trisodium citrate was drawn from various donors who had not taken aspirin for at least 10 days. All labware was either plastic or siliconized glass. Whole blood was centrifuged at $160 \times g$ for 20 min at 20°C. The supernatant was removed and centrifuged at $800 \times g$ for 10 min at 20°C. The platelet pellet was gently resuspended once in 10 ml wash buffer (0.15 M NaCl/0.01 M Tris/0.03 M glucose/0.001 M EDTA (pH 7.4)) and recentrifuged at $800 \times g$ for 10 min at 20°C. The final pellet was resuspended in the desired volume of Tyrodes buffer (0.138 M NaCl/2.7 mM KCl/ 12 mM sodium bicarbonate/ 0.36 mM sodium phosphate/0.49 mM $MgCl_2$/5.5 mM glucose/1.8 mM $CaCl_2$ (pH 7.4)).

Transport assay. [^{3}H]Putrescine uptake was assayed basically utilizing the method of Wilkins et al. [14]. The platelet suspension in Tyrodes buffer was preincubated at 37°C for 15 min in a shaking water-bath. To the platelet suspension [^{3}H]-putrescine was added to the required final concentration. The platelets were incubated at 37°C in a shaking water-bath. At the indicated times, an aliquot was removed, filtered on a Millipore filter (Type HA, 25 mm, 0.45 μm pore size), and rapidly washed with 5 ml ice-cold wash buffer (0.12 M NaCl/0.03 M Tris/3 mM EDTA/5 mM glucose/1 mM putrescine (pH 7.4)) The filters were dried and dissolved in 10 ml ACS scintillation fluid (Amersham) and counted. The blank for putrescine uptake at 37°C was determined by incubating platelets at 0°C and assaying as described. This blank will account for nonspecific binding to the filter, and any putrescine trapped in the extracellular place. This blank could not be used for Fig. 1. Only putrescine binding to the filter was taken into account in FIg. 1.

Investigation of energetics of putrescine uptake. For the following study, a Hepes buffer comprising 20 mM Hepes/5.5 mM glucose/0.5 mM $MgCl_2$ (pH 7.4) was used as the stock buffer with the following additions of salts to the concentrations indicated below. *N*-Methyl-D-glucamine was used to replace Na^+ or K^+, since it has no deleterious effects on platelet morphology, platelet function, α-2-adrenergic receptor binding and adenylate cyclase activity [15]. To determine the effects of a Na^+ gradient (in > out), platelets were preincubated in the Hepes buffer containing 37.5 mM NaCl and 2.7 mM KCl for 45 min at 37°C. The platelets were then transferred to a large volume of the same buffer without Na^+, containing 3 μM [^{3}H]putrescine (1.56 Ci/mmol) and assayed for putrescine uptake as described above. The control platelets were preincubated in the same buffer and then transferred to a buffer containing 150 mM NaCl and 2.7 mM KCl to create a Na^+ gradient (out > in). Dependence on a K^+ gradient (in > out) was determined in a similar fashion, except the platelets were first preincubated in Hepes buffer containing 150 mM NaCl and 2.7 mM KCl. Platelets were then transferred to the same buffer without K^+ and containing 25 μM valinomycin. Control platelets were preincubated in Hepes buffer containing 150 mM NaCl and 2.7 mM KCl, and then transferred to buffer with 0.68 mM KCl and 150 mM NaCl, to create a K^+ gradient (in > out). the 45-min preincubation prior to assay is performed to equilibrate intracellular and extracellular salts and temperature after the platelet wash.

Partial purification of a putrescine transport protein. One unit (50 ml) of platelet concentrate was obtained from New Jersey Blood Services and used within 3 days after drawing. the platelet membranes were isolated using a modification of a previously published procedure [16]. The platelet-rich plasma was centrifuged at $160 \times g$ for 20 min to remove red blood cells. The platelet-rich plasma was then centrifuged at $800 \times g$ for 10 min at 20°C and the pellet was washed twice with 40 ml buffer (0.108 M NaCl/21 mM trisodium citrate/1 mM $MgCl_2$/2 mM sodium bicarbonate/28 mM

glucose/3 mM EDTA (pH 6.5)). The final pellet was suspended in 2 ml buffer B (0.108 M NaCl/21 mM trisodium citrate/5 mM EDTA/0.4 mM phenylmethylsulfonyl fluoride/0.5 mM *N*-carbobenzoxy-L-glutamyl-L-tyrosine (pH 6.5)). The platelets were freeze-thawed four times and the membranes were extracted with 5% Triton X-100 to yield a final concentration of Triton X-100 of 1%. The samples were then sonicated on ice, two times, 30 s each with the microtip of a LabSonic sonicator, and then centrifuged at $20000 \times g$ for 20 min at 4°C to remove the insoluble cytoskeleton. The supernatant (membrane fraction) was diluted with an equal volume of buffer B to bring the Triton X-100 concentration to 0.5%. The membranes were then chromatographed on an affinity column of putrescine coupled to activated CH-Sepharose 4B [17]. The column was run at 4°C and the protein was eluted with a step gradient using the following buffers. Unbound protein was first eluted with buffer B containing 0.5% Triton X-100. Protein which was nonspecifically bound was eluted with the same buffer containing 39 mM spermidine and 39 mM spermine. Finally, the transport protein was eluted with buffer B plus 0.5% Triton X-100 containing 0.315 M putrescine. Three bed volumes of each buffer were used.

Differential labeling experiment The differential labeling experiment was performed essentially using the method of Fox and Kennedy [18]. Briefly, 50 ml platelets in plasma were washed twice as described above, and suspended in Tyrodes buffer to $5 \cdot 10^9$ platelets/ml. The experimental sample was incubated with 10 mM putrescine for 20 min at 37°C in a shaking water-bath. After incubation, unlabeled *N*-ethylmaleimide was added to a final concentration of 2 mM, and incubated an additional 20 min, at 37°C. The platelet suspension was washed first in Tyrodes buffer containing 10 mM putrescine and then in Tyrodes buffer without putrescine. The platelets were then incubated with 1 mM *N*-[^{14}C]ethylmaleimide for 20 min at 37°C. After incubation, the platelets were washed and the membranes were solubilized as described above. The control platelets were simply treated with 1 mM *N*-[^{14}C]ethylmaleimide and prepared in the same manner as the experimental platelets.

Effects of N-ethylmaleimide on putrescine uptake. The control platelets were prepared and assayed for putrescine uptake using 1 μM [^{3}H]putrescine and removing aliquots at various times to yield initial rates of uptake. *N*-Ethylmaleimide-treated platelets were treated with 0.2 mM *N*-ethylmaleimide for 10 min, washed once in Tyrodes buffer containing 0.1 mM putrescine and then in buffer without putrescine. After washing, initial rates of putrescine uptake were assayed. The putrescine-blocked samples were first treated with 100 μM putrescine for 10 min then with 0.2 mM *N*-ethylmaleimide for an additional 10 min. The platelets were then treated as indicated above.

Polyacrylamide gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis was performed under reducing conditions in 9% slab gels according to the method of Laemmli [19]. Samples containing any of the polyamines were first dialyzed agains 1 M NaCl containing 0.5% Trition X-100 The samples were then solubilized in a buffer comprising 0.5 M Tris-HCl (pH 6.8)/6% SDS/2-mercaptoethanol/50% glycerol/0.05% Bromophenol blue. Gels were either silver-stained [20] or stained with Coomasie blue. Fluorography was performed according to instructions for the use of Enhance (New England Nuclear). The gels were dried after treatment with Enhance and then exposed to Kodak X-OMat-AR type film at −80°C for 15 days. The film was developed with a Kodak developer type D-19.

Results

General characteristics of putrescine uptake in platelets

The study of putrescine transport into human platelets, requires determination of platelet internal volumes. A platelet volume of 0.73 μl/10^8 platelets was calculated by loading platelets with L-[^{3}H]glucose which is permeable and not metabolized. This is similar to the value of 0.52 μl/10^8 platelets reported by Wilev et al. [21]. The concentration of intracellular putrescine is calculated to be approx. 2.7-times higher then extracellular concentrations, and thus appears to represent active accumulation against a concentration gradient. To estimate binding of putrescine to platelet proteins, platelets were incubated 2 h with labeled putrescine and then extracted three times with 10% trichloroacetic acid. No more than 5% of the

labeled putrescine was retained in the acid-insoluble pellet. Paper chromatography of ethanol extracts of platelets incubated with [^{3}H]putrescine for 2 h indicated that the putrescine was not metabolized [8].

The K_m for putrescine transport was determined to be 4.42 μM with $V_{max} = 0.046$ nmol putrescine/10^{10} platelets per min, using an Eadie-Hofstee plot (data not shown). The binding and passive diffusion components of total uptake were subtracted from total uptake rates using a blank at 0°C. Platelet transport is slow compared to uptake of amino acids; i.e., the V_{max} for amino acid uptake is approx. 20–200-times greater than the V_{max} for putrescine uptake.

Table I shows the effects of various compounds on putrescine uptake. The other polyamines, spermidine and spermine, at 500 μM, only slightly inhibit uptake, indicating that the transport process is fairly specific for putrescine. The amino acids L-phenylalanine and L-glutamine which are transported by energy-dependent processes [22], have no significant effect on putrescine transport. L-Tyrosine which simply diffuses into the platelet, also has no effect on putrescine uptake. Amino acid uptake is therefore distinct from putrescine uptake. Ca^{2+} has no observable effect on putrescine uptake. Since the serotonin transport system in platelets does not show absolute structural specificity and transports other amines [23], imipramine, an inhibitor of serotonin uptake at the plasma membrane, was tested and shown to effectively inhibit putrescine transport at 50 μM. Reserpine, an inhibitor of serotonin transport into dense granules, has no effect on putrescine transport. The inhibition by imipramine suggests that the putrescine uptake system may be similar to or identical with the serotonin transporter of platelets [25–28].

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON PUTRESCINE TRANSPORT IN HUMAN PLATELETS

All compounds were preincubated with platelets for 20 min before assaying with 5 μM [^{3}H] putrescine. Uptake was measured after 60 min of incubation at 37°C Data presented are the mean ± S D. of 3–5 separate experiments

Compound	Concn (μM)	% of Control
Spermidine	500	68 ± 14
Spermine	500	71 ± 16
L-Tyrosine	100	110 ± 6
L-Phenylalanine	100	93 ± 13
L-Glutamine	100	108 ± 8
Calcium-free buffer		110 ± 9
Calcium	6-times control	97 ± 23
Imipramine	50	58 ± 11
Reserpine	100	100 ± 5

Studies on the energetics of putrescine transport

The uptake of putrescine appears to be energy-dependent, since it is partially inhibited at 0°C and also inhibited by the uncoupler 2,4-dinitrophenol (Fig. 1). One reason why only partial inhibition is seen in this experiment is that passive diffusion and putrescine entrapment in the platelet pellet may contribute to total uptake, since the blank corrections applied accounts only for non-specific binding to the filter. Experiments designed to identify driving forces for putrescine uptake were undertaken despite difficulties inherent in the use of whole cells to study the transport; ion concentrations are difficult to regulate and cyto-

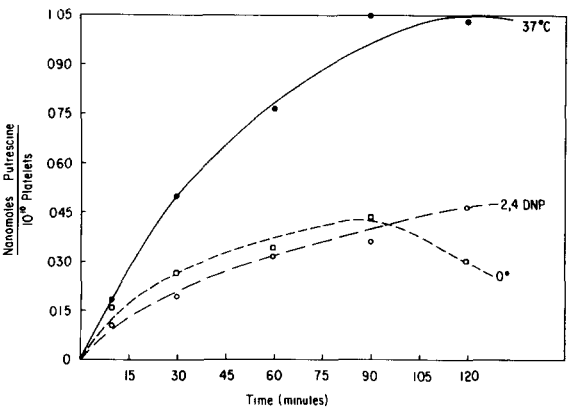

Fig 1 Effect of 2,4-dinitrophenol (2,4-DNP) and incubation at 0°C on putrescine uptake To 1 4 ml of a platelet suspension in Tyrodes buffer (approx. 5 10^8 platelets/ml), either 2,4-dinitrophenol was added to 0 5 mM and incubated for 15 min, or the platelet suspension was incubated at 0°C After incubation, [^{3}H]putrescine (0 25 Ci/mmol) was added to a final concentration of 5 μM 0.25-ml aliquots were removed at the indicated times and assayed as described in Materials and Methods Data plotted represent the mean of three experiments Putrescine binding to the filter was used as the blank for this experiment only

plasmic organelles complicate interpretation of results.

Platelets were assayed for putrescine uptake under conditions which do not activate platelets [29–30]. Putrescine transport is not dependent on a Na^+ gradient (Fig. 2); this is confirmed by the fact that the ionophore gramicidin (1 μM) also does not affect uptake (data not shown). The ionophore valinomycin which dissipates the K^+ gradient does not affect transport (Fig. 2), and Table II shows that there is no significant difference between samples A (without a K^+ gradient), or sample D (with a K^+ gradient). Putrescine uptake is partially dependent on a H^+ gradient, since the protonophore FCCP inhibits transport (Table II, sample E). Additional evidence supporting a transport role for the pH gradient is seen in Fig. 3. Putrescine transport is stimulated in response to a pH gradient (acid-inside). However, since FCCP and 2,4-dinitrophenol only partially inhibit uptake, the pH gradient is not the sole driving force for uptake. The effects of increasing the net negative-inside membrane potential [25] are seen in Table II, sample C. There is a significant increase in transport induced by NaSCN. Sample B contains both valinomycin and FCCP which should dissipate both the proton gradient and membrane potential, and shows the highest inhibition; however, complete inhibition is not observed. Therefore, it appears that the putrescine uptake is highly complex and may involve mem-

TABLE II

STUDY OF THE DRIVING FORCE(S) FOR PUTRESCINE UPTAKE

All samples were preincubated in 20 mM Hepes/5.5 mM glucose/0.5 mM $MgCl_2$/20 mM NaCl/130 mM KCl (pH 7.4) at 37°C for 45 min. 0.15 l of the platelet suspension was then diluted with 1.4 ml of the second buffer. All buffers consisted of 20 mM Hepes, 5.5 mM glucose, 0.5 mM $MgCl_2$ (pH 7.8), with the indicated additions of salts or inhibitors (A,B), 20 mM NaCl, 130 mM KCl, (C) 20 mM NaSCN, 130 mM KCl; (D,E), 20 mM NaCl, 5 mM KCl, 125 mM *N*-methyl-D-glucamine. Putrescine transport was assayed using 2 μM [^{3}H]putrescine (2.73 Ci/mmol) determined after 15 min incubation at 37°C. Data are the mean S.D. of three experiments.

	Uptake after 15 min (cpm)
No K^+ gradient	
A control	1246 ± 205
B 1 μM valinomycin, 40 μM FCCP	572 ± 56
C 20 mM NaSCN	1759 ± 267
With K^+ gradient	
D control	1150 ± 30
E 40 μM FCCP	871 ± 20

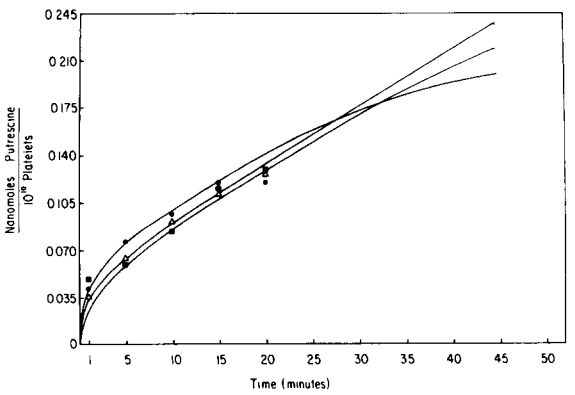

Fig. 2. Study of the forces driving putrescine uptake. The platelets were preincubated in 0.5 ml Hepes buffer with various salts or inhibitors as described in Materials and Methods. After a 45-min preincubation, 0.5 ml of the platelet suspension was diluted with 4.5 ml of the second buffer which contained 3 μM [^{3}H]putrescine (1.56 Ci/mmol). The platelets were incubated at 37°C and 0.25-ml aliquots were removed at the indicated times and assayed for putrescine uptake. Control (△), 25 μM valinomycin (■), Na^+-depleted buffer (●).

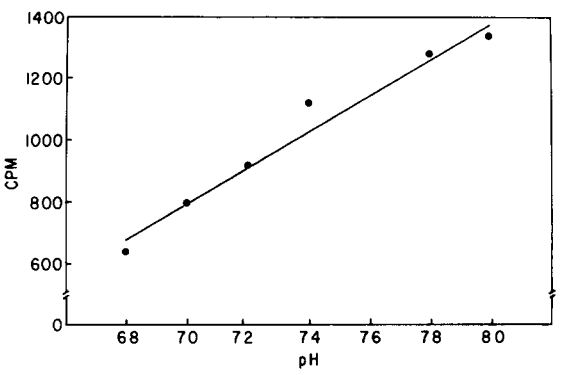

Fig. 3. Determination of H^+ dependence for putrescine transport. Platelets ($3 \cdot 10^9$ platelets/ml) were preincubated for 45 min in a buffer comprising of 20 mM Hepes/5.5 mM glucose/0.5 mM $MgCl_2$/150 mM NaCl/2.7 mM KCl (pH 7.4) at 37°C. After incubation, 0.1 ml of the platelet suspension was added to 0.42 ml Hepes buffer at the indicated pH, and incubated an additional 10 min. [^{3}H]Putrescine was then added to a final concentration of 1 μM and initial uptake was measured after a 2-min incubation

Fig 4 Silver-stained gel of samples from putrescine affinity column 9% SDS-polyacrylamide gel electrophoresis under reducing conditions. Lane 1, molecular weight ($\times 10^3$) markers, carbonic anhydrase (29), ovalbumin (45), bovine serum albumin (66), phosphorylase B (97 4), β-galactosidase (116) Lane 2, actin (5 μg); lane 3, platelet whole membranes (60 μg) Proteins eluted with lane 4, buffer B (20 μg), lane 5, buffer B with spermidine/spermine (20 μg); lane 6, buffer B with putrescine (4 μg)

brane potential (negative-inside) and pH gradient (acid-inside), and other as yet unidentified factors.

Partial purification of a putrescine transport protein

A simple, one-step purification utilizing affinity chromatography was perfected. Platelet membranes were solubilized with Triton X-100 and chromatographed on the putrescine-Sepharose affinity column as described in Materials and Methods. Spermidine and spermine were used to remove any protein which was nonspecifically bound to the cationic affinity column, since the transport data indicated that they had a low affinity for the putrescine transporter. Using buffers at pH 6.5, we obtained the greatest purification of the putrescine transport protein. Comparing lane 6 with lane 5 (Fig. 4), a protein with an apparent molecular weight of 65000 is a prominent protein band specifically eluted with putrescine. The protein in lane 6 with an apparent molecular weight of 45000 has been identified as actin, since it comigrates with pure actin on the polyacrylamide gel (Fig. 4), and inhibits deoxyribonuclease I (data not shown) [31]. A protein with an apparent molecular weight of 55000 also elutes with putrescine. Both the 55 and the 65 kDa proteins may be involved in putrescine transport. Differential *N*-ethylmaleimide labeling was also used to identify proteins involved in putrescine transport.

Differential N-ethylmaleimide labeling

Since covalent affinity labels for putrescine are not readily available, a differential labeling technique was employed; *N*-ethylmaleimide was used in the presence and absence of putrescine to label the putrescine-binding protein. As can be seen in Fig. 5, there are three major bands (cross-hatched) where the labeling in the experimental group exceeds that in the corresponding band in the control. The protein indicated by the arrow has an apparent molecular weight of 65000. This band

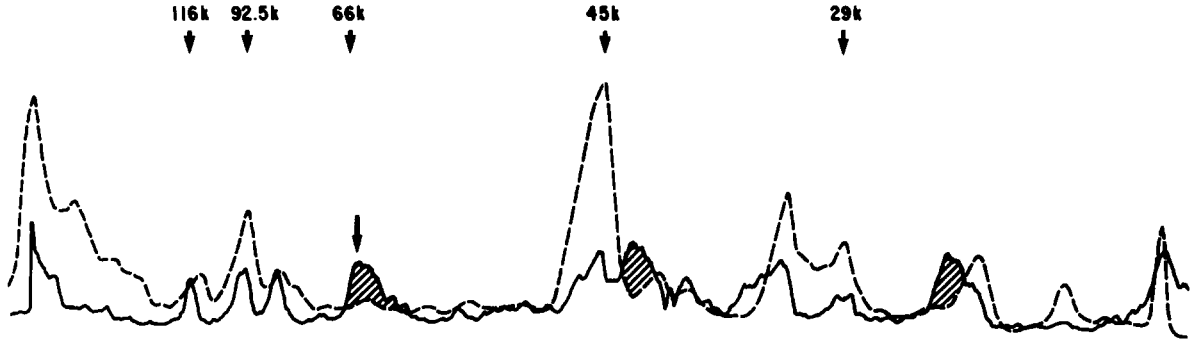

Fig 5 Differential labeling of putrescine-binding proteins Electrophoresis through a 9% SDS gel was run under reducing conditions using equal amounts of protein for experimental and control samples Densitometric scan of the fluorogram Experimental (———), control (- - - - - -)

shows the highest percentage enhancement of labeling within the experimental group. Two additional proteins with molecular weights of 45 000 and 23 000 are also labeled. We assign the 45 kDa protein band to actin, because of the affinity-column results and because actin is known to strongly bind putrescine.

To determine whether *N*-ethylmaleimide binds to the protein involved in putrescine transport, the effect of *N*-ethylmaleimide on putrescine uptake was studied. 200 μM *N*-ethylmaleimide inhibits the initial uptake of putrescine by $60 \pm 2\%$ ($X \pm$ S.D.). When the membranes were treated with 100 μM putrescine, there was partial putrescine protection with only a $29 \pm 13\%$ ($X \pm$ S.D.) inhibition of uptake compared to the control. Putrescine protection is not complete possibly because of non-specific effects upon other membrane proteins. Correlating the results from the affinity column with the differential labeling experiment, only the 65 kDa protein is eluted by putrescine from the affinity column and is specifically labeled in the differential label experiment. We conclude that this protein is the putrescine-transport protein, or at least the putrescine-binding portion of the transporter. However, the 23 and 55 kDa proteins may also be involved in the uptake process.

Conclusions

Polyamines have been shown to inhibit platelet aggregation [4], even at micromolar levels [5]. This appears not to be due to simple competition with calcium [6]. Polyamines have many effects on membranes, including stabilization of erythrocyte membranes and decrease of membrane protein lateral mobility [12,13]. These effects only occur when the polyamines are on the cytoplasmic side of the membrane, indicating that transport of the polyamine is required. This was the basis for studying putrescine transport into platelets.

This study has characterized the uptake of putrescine into human blood platelets at micromolar levels which are found in the plasma. Although the platelet is fully differentiated, does not proliferate and shows minimal protein synthesis, it still has the ability to transport putrescine. These preliminary studies suggest putrescine uptake into platelets may be dependent upon a pH gradient (acid-inside) and a negative internal membrane potential. Further studies using membrane vesicles are indicated to clearly define the driving force(s) for uptake. Putrescine uptake in platelets appears to have some similarities to the complicated serotonin transport system in platelets studied by Rudnick et al [25–28]. Among the similarities are membrane-potential dependence, inhibition by imipramine and little or no effect of reserpine; however, we have not observed effects of Na^+ gradient nor of K^+.

Some of the proteins identified in this study clearly are involved in putrescine uptake. Putrescine-binding proteins have been partially purified using affinity chromatography. Rotman and Pribluda [32] have identified a serotonin carrier ($M_r = 65\,000$) in platelets using a photoaffinity label. In addition, they also saw labeling of a 60 kDa, 45 kDa and two low molecular weight proteins. The 45 kDa protein labeled in both our study and the study of Rotman and Pribluda [32] appears to be actin, and probably is not involved in transport. The labeling of the 65 kDa protein is similar in both studies. It is quite possible that the transport system which we have studied is identical to the serotonin transport system in view of the similarities in specific labeling of membrane proteins and the inhibition by imipramine. The energetics of uptake may be postulated to differ because of the charge difference between serotonin (+1) and putrescine (+2). Further characterization of the polyamine transport process and determination of whether the two systems are identical should be aided by additional studies of the proteins identified in this paper.

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