Dependence of mammalian putrescine and spermidine transport on plasma-membrane potential: identification of an amiloride binding site on the putrescine carrier

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The mechanism of mammalian polyamine transport is poorly understood. We have investigated the role of plasma-membrane potential ($\Delta\Psi_{pm}$) in putrescine and spermidine uptake in ZR-75-1 human breast cancer cells. The rate of [^{3}H]putrescine and [^{3}H]spermidine uptake was inversely correlated to extracellular [K^+] ($[K^+]_o$) and to $\Delta\Psi_{pm}$, as determined by the accumulation of [^{3}H]tetraphenylphosphonium bromide (TPP). Inward transport was unaffected by a selective decrease in mitochondrial potential ($\Delta\Psi_{mit}$) induced by valinomycin at low $[K^+]_o$, but was reduced by $\approx 60\,\%$ by the rheogenic protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which rapidly ($\leqslant 15$ min) collapsed both $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$. Plasma-membrane depolarization by high $[K^+]_o$ or CCCP did not enhance putrescine efflux in cells pre-loaded with [^{3}H]putrescine, suggesting that decreased uptake caused by these agents did not result from a higher excretion rate. On the other hand, the electroneutral K^+/H^+ exchanger nigericin (10 μM) co-operatively depressed [^{3}H]TPP, [^{3}H]putrescine and [^{3}H]spermidine uptake in the presence of ouabain. Suppression of putrescine uptake by nigericin + ouabain was Na^+-dependent, suggesting that plasma-membrane repolarization by the electrogenic Na^+ pump was required upon acidification induced by nigericin, due to the activation of the Na^+/H^+ antiporter. The sole addition of 5-*N*,*N*-hexamethylene amiloride, a potent inhibitor of the Na^+/H^+ antiporter, strongly inhibited putrescine uptake in a competitive fashion [K_i 4.0 ± 0.9 (S.D.) μM], while being a weaker antagonist of spermidine uptake. The potency of a series of amiloride analogues to inhibit putrescine uptake was clearly different from that of the Na^+/H^+ antiporter, and resembled that noted for Na^+ co-transport proteins. These data demonstrate that putrescine and spermidine influx is mainly unidirectional and strictly depends on $\Delta\Psi_{pm}$, but not $\Delta\Psi_{mit}$. This report also provides first evidence for a high-affinity amiloride-binding site on the putrescine carrier, which provides new insight into the biochemical properties of this transporter.

INTRODUCTION

Putrescine (Put) and the natural polyamines, spermidine (Spd) and spermine (Spm), are essential for eukaryotic cell growth [1,2]. These compounds can be synthesized from L-ornithine and *S*-adenosylmethionine by most cell types, but can also be accumulated from extracellular sources via at least one specific transport system [3,4]. The uptake of exogenous polyamines, especially those originating from the gastrointestinal microflora and dietary sources, is considered as a major obstacle to anti-cancer therapies aimed at depleting polyamines through the use of specific inhibitors of biosynthesis such as α-difluoromethyl-ornithine [4,5]. This problem is compounded by the fact that tumour cells exhibit increased polyamine transport activity as compared with normal tissues [4,6], and that polyamine depletion alleviates normal feedback repression of transport by endogenous polyamines and leads to a compensatory increase in uptake activity [4,7–10]. Although genes encoding three different types of carriers for Put and/or Spd have been cloned and characterized in *Escherichia coli* [3], the molecular identities of the eukaryotic polyamine transporters have remained elusive. The ornithine decarboxylase antizyme, in addition to its role in the regulation of ornithine decarboxylase turnover, is a mediator of the feedback repression of polyamine transport in mammalian cells through an unknown mechanism [10].

Although no mammalian polyamine carrier has yet been molecularly characterized, its physiological properties have been extensively studied in several cell types. In the vast majority of models studied to date, the uptake of Put and polyamines is a saturable process and is steeply energy-dependent [4,7,11–13]. Diamine and polyamine transport is not Na^+-dependent, and is inhibited by high concentrations of monovalent cations such as Na^+, Li^+, choline and *N*-methyl-D-glucamine in intact cells [11–15] as well as in intestinal brush-border membrane vesicles [16,17]. On the other hand, extracellular divalent cations such as Ca^{2+} and Mg^{2+} are essential for Put and Spd transport, apparently through tight binding to exofacial sites on the carrier or plasma membrane [11,17]. A marked decrease of Put and Spd uptake at $pH < 7.0$ has also been noted in both intact breast cancer cells [11] and in brush-border membrane vesicles [18], but the inhibitory mechanism is still unclear. The number of transporter types involved in the uptake of Put, Spd and Spm is still much debated. It is nevertheless clear that diamine and polyamine carriers share common organizational features, as suggested by

Abbreviations used: $\Delta\Psi_{pm}$, plasma-membrane potential; $\Delta\Psi_{mit}$, mitochondrial potential; $[K^+]_o$, extracellular K^+ concentration; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; HMA, 5-*N*,*N*-hexamethylene amiloride; pH_i, intracellular pH; Put, putrescine; Spd, spermidine; Spm, spermine; TPP, tetraphenylphosphonium bromide.

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the mutual competition between Put and the polyamines for inward transport [4,9,15,19] and by the fact that all mammalian polyamine transport mutants thus far isolated are equally deficient in the uptake of Put, Spd and Spm [20–23].

Although the Na^+ electrochemical gradient is not the driving force for diamine and polyamine transport, there is preliminary evidence that the plasma-membrane potential ($\Delta\Psi_{pm}$) might govern the rate of uptake of these compounds in mammalian cells [11,19] as in *E. coli* [24]. Moreover, Spd efflux from *Xenopus laevis* oocytes proceeds via a voltage-dependent, electrodiffusive process [25]. In support of an electrogenic component in mammalian polyamine transport, increases in extracellular K^+ ($[K^+]_o$) non-competitively inhibited Put and Spd transport [11], whereas protonophores such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and the Ca^{2+}/H^+ exchanger A23187 strongly depressed the uptake of these compounds [11,19]. However, although $\Delta\Psi_{pm}$ in epithelial cells is mainly due to a K^+ transmembrane gradient [26–28], imposition of an inside-negative K^+ diffusion potential in intestinal brush-border membrane vesicles did not influence the rate of polyamine uptake, whereas it increased that of amphiphilic amines such as tryptamine and chlorpromazine [16]. Moreover, a high mitochondrial membrane potential ($\Delta\Psi_{mit}$) favours the internalization and binding of lipophilic cations [27]. Since mitochondria can accumulate di- and poly-amines in a $\Delta\Psi_{mit}$-dependent manner [29], and the compartmentalization of newly internalized polyamines has not yet been determined, the possibility that uptake of polyamines by mitochondria with an elevated $\Delta\Psi_{mit}$ represents a substantial fraction of total polyamine accumulation cannot be ruled out.

To assess in more detail the importance of membrane potential in Put and Spd transport, we have measured the effect of various manipulations of $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$ on the uptake of tetraphenylphosphonium bromide (TPP), a lipophilic cation that is accumulated by mammalian cells as a function of both $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$ [30], in parallel with determinations of Put and Spd influx. We used for this purpose ZR-75-1 human breast cancer cells, which exhibit elevated Put and Spd transport activities that are under complex hormonal and feedback regulation [9,11]. The present results clearly demonstrate that Put and Spd uptake in these cells selectively responds to changes in $\Delta\Psi_{pm}$, but not $\Delta\Psi_{mit}$. Depolarization caused by the simultaneous stimulation of Na^+/H^+ exchange and inhibition of the Na^+/K^+-ATPase completely abolishes Put uptake, and substantially decreases Spd transport. Surprisingly, we also demonstrate that 5-alkylated amiloride analogues are potent competitive inhibitors of Put uptake, with a pharmacological profile similar to that found for Na^+-dependent co-transport proteins.

MATERIALS AND METHODS

Materials

[2,3-^{3}H(N)]Put dihydrochloride (4.1×10^4 Ci/mol), [1,8-^{3}H(N)]Spd trihydrochloride (1.52×10^4 Ci/mol) and [*phenyl*-^{3}H]TPP (3.84×10^4 Ci/mol) were obtained from DuPont–New England Nuclear (Lachine, Québec, Canada). Foetal bovine serum was purchased from Gibco-BRL Life Technologies (Burlington, Ontario, Canada). Unlabelled TPP was obtained from Aldrich (Milwaukee, WI, U.S.A.). 5-(*N*-ethyl,*N*-isopropyl)amiloride and 5-*N*,*N*-dimethyl amiloride were obtained from Molecular Probes (Eugene, OR, U.S.A.). Benzamil was purchased from Research Biochemicals (Natick, MA, U.S.A.). Amiloride, valinomycin, nigericin, ouabain, CCCP, Put dihydrochloride, Spd trihydrochloride, Spm tetrahydrochloride, 5-*N*,*N*-hexamethylene amiloride (HMA), as well as growth media, tissue-culture supplements and other biochemical reagents were purchased from Sigma. Stock solutions of nigericin, valinomycin, CCCP, TPP and amiloride or amiloride analogues were prepared in EtOH, and the final EtOH concentration in cell media did not exceed 0.2 % (v/v).

Radiometric determination of Put, Spd and TPP uptake

Before experiments, ZR-75-1 human breast cancer cells (obtained from the American Type Culture Collection) were plated in 24-well Falcon tissue-culture plates at 3×10^4 cells/well and grown for 3–4 days in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 15 mM Hepes and antibiotics, with fresh medium being replaced every other day [9,11]. Put- and Spd-uptake assays were performed in 400 μl of RPMI 1640 medium (pre-warmed at 37 °C) containing the indicated test compounds, plus 20 μM [^{3}H]Put (20 Ci/mol) or 5 μM [^{3}H]Spd (20 Ci/mol). In experiments where [^{3}H]TPP uptake was determined in parallel, 5 μM unlabelled TPP was also included in the uptake medium; the presence of TPP did not significantly influence Put or Spd uptake. After incubation for 20 min (unless otherwise indicated) at 37 °C under a 5 % CO_2 atmosphere, the radioactive substrate solution was removed, and internalization of the radioactive substrate, as well as non-specific uptake, were then determined as described [9]. Total cellular DNA content was determined in parallel culture wells with 3,5-diaminobenzoic acid [31]. For the determination of kinetic parameters of transport, the substrate concentration was varied by adding increasing amounts of non-radioactive substrate to a fixed concentration of [^{3}H]Put or [^{3}H]Spd, and the K_m and V_{max} values were determined by Lineweaver–Burk analysis.

TPP uptake was determined as above for Put and Spd, using 5 μM [^{3}H]TPP (40 Ci/mol) for 20 min, unless otherwise indicated. At the end of the incubation period, cells were washed four times with 1 ml of ice-cold, Ca^{2+}-, Mg^{2+}-free PBS (2.7 mM KCl, 1.5 mM KH_2PO_4; 8.1 mM Na_2HPO_4 and 137 mM NaCl). Non-specific [^{3}H]TPP binding did not exceed 3 % of total cellular radioactivity after 20 min, and was determined by adding ice-cold uptake assay solution for 15 s to cells pre-incubated with 1 μM CCCP in NaCl-free RPMI 1640 medium containing 108 mM KCl.

Na^+ and K^+ substitution experiments

The ionic composition of incubation media was varied by modifying the formulation of RPMI 1640 medium used for the uptake assay. A RPMI 1640-based medium was first reconstituted from all its various constituents, minus NaCl, KCl, $NaHCO_3$, and Na_2HPO_4, and was buffered with 20 mM Tris/HCl, (pH 7.4 at 37 °C) [medium A$^-$]. Complete medium A was then reconstituted by adding 103 mM NaCl/5.4 mM KCl/24 mM $NaHCO_3$.

For experiments designed to study the dependence of Put and Spd uptake on $[K^+]_o$, $NaHCO_3$ (24 mM) was restored to medium A$^-$, and KCl was added at the desired concentration (up to 108 mM). NaCl was then added to its standard concentration of RPMI 1640 (103 mM), minus the molar equivalent of added KCl; when the KCl concentration was below its normal value in RPMI 1640 (5.4 mM), NaCl was added so that the sum of NaCl and KCl concentrations was constant (108.4 mM).

To study the effect of extracellular Na^+, KCl was restored to medium A$^-$ at the standard concentration (5.4 mM), and $NaHCO_3$ was replaced with an osmotically equivalent concentration of sucrose (48 mM). NaCl was added at the desired concentration, and the osmolality was set constant at

325 mOsmol/kg by adding sucrose. Uptake assays were then conducted in a CO_2-free incubator at 37 °C.

Determination of [^{3}H]Put efflux

In order to measure net [^{3}H]Put export, ZR-75-1 cell monolayers were pre-incubated for 20 min at 37 °C in the presence of 20 μM [^{3}H]Put in 400 μl of RPMI 1640 medium, and then washed three times with 1 ml of RPMI 1640. After the final rinsing step, cells were incubated for 5, 30, 60 or 120 min with 1 ml of complete medium A (except for incubation at high $[K^+]_o$) supplemented as indicated, before determination of radioactivity in a 400 μl aliquot of extracellular medium. Total intracellular radioactivity was also measured in parallel as for uptake assays [9], and the amount of [^{3}H]Put excreted was expressed as a percentage of total cellular radioactivity (extra- + intra-cellular). Medium used for incubation with 108 mM KCl was prepared from medium A^- as described above for K^+ substitution experiments.

Statistical analysis and other methods

Unless otherwise indicated, comparisons between two sample means were made by unpaired Student's *t* tests with analysis of variance. Intracellular pH determinations with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein were performed as described [32], using a SLM 8000 spectrofluorometer (SLM-Aminco, Champagne, IL, U.S.A.).

RESULTS

Selective dependence of Put and Spd transport on $\Delta\Psi_{pm}$

To assess better the contribution of $\Delta\Psi_{pm}$ as a factor driving Put and Spd transport in ZR-75-1 cells, we determined the effect of iso-osmotic replacement of NaCl with KCl on the uptake of [^{3}H]Put and [^{3}H]Spd, as well as [^{3}H]TPP, a probe of both $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$ [30]. The $\Delta\Psi_{pm}$ in epithelial cells is mainly determined by the K^+ diffusion potential [26,27], and should thus rapidly respond to changes in $[K^+]_o$. As shown in Figure 1A, [^{3}H]TPP accumulation was rapidly reduced by 50 % in the presence of high $[K^+]_o$ (100 mM) and reached a plateau roughly corresponding to that observed in the presence of the K^+ ionophore valinomycin (10 μM). At normal $[K^+]_o$ (5.6 mM), valinomycin selectively and fully depolarizes mitochondria at the concentration used [33], and, thus, there were approximately equal contributions of $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$ in determining total [^{3}H]TPP uptake under standard conditions. The selectivity of plasma and mitochondrial membrane depolarization by high $[K^+]_o$ and valinomycin was further supported by the additive effect of both agents on [^{3}H]TPP uptake. Thus the decrease in [^{3}H]TPP uptake observed in the presence of valinomycin at physiological $[K^+]_o$ is a valid estimate of the contribution of $\Delta\Psi_{mit}$ in favouring [^{3}H]TPP accumulation in ZR-75-1 cells. Valinomycin had no effect on Put accumulation and even increased this parameter at late time points, whereas 100 mM KCl strongly and rapidly depressed this parameter (Figure 1B), suggesting that Put transport is insensitive to $\Delta\Psi_{mit}$, but is selectively controlled by the $\Delta\Psi_{pm}$ established by the K^+ diffusion potential.

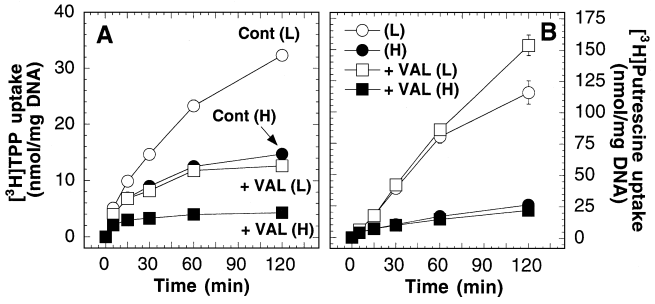


Figure 1 Effect of extracellular K^+ ($[K^+]_o$) and valinomycin on [^{3}H]TPP and [^{3}H]Put uptake in ZR-75-1 cells

At time zero, cells grown in standard growth medium were transferred to 400 μl of medium A^- (supplemented with 24 mM $NaHCO_3$) containing 10 μM valinomycin (VAL, solid symbols) or the ethanol vehicle (0.01 %, v/v) (Cont, plain symbols) and either 5.4 mM KCl (low $[K^+]_o$, L) (○, ●) or 100 μM KCl (high $[K^+]_o$, H) (□, ■), in the presence of either 5 μM [^{3}H]TPP (**A**) or 20 μM [^{3}H]Put (**B**). The ionic strength was kept constant by adjusting the NaCl concentration (see the Materials and methods section). Cells were incubated for the indicated time interval before determination of intracellular radioactivity. Values represent the means ± S.D. of triplicate determinations from three independent experiments.

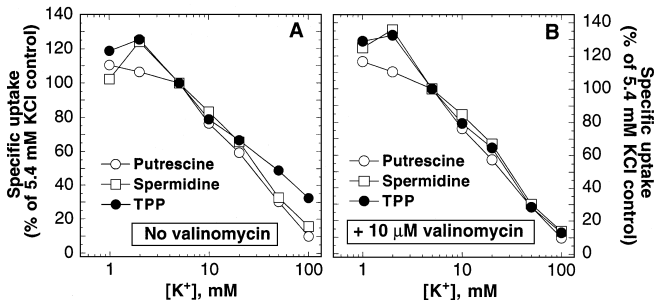


Figure 2 Dependence of the rate of [^{3}H]Put, [^{3}H]Spd and [^{3}H]TPP uptake on $[K^+]_o$

ZR-75-1 cells were incubated in medium containing the indicated $[K^+]_o$ (as KCl) (see the legend to Figure 1), in the absence (**A**) or presence (**B**) of 10 μM valinomycin and 20 μM [^{3}H]Put (○), 5 μM [^{3}H]Spd (●) or 5 μM [^{3}H]TPP (□). Intracellular radioactivity was determined after a 20-min incubation period. Values represent the means ± S.D. of triplicate determinations.

As illustrated in Figure 2A, there was a log-linear relationship between $[K^+]_o$ and the velocity of Put as well as Spd uptake. This dependence was closely correlated to the parallel change in [^{3}H]TPP uptake at $[K^+]_o \leqslant 20$ mM, even at [KCl] below the standard value (5.4 mM) present in growth media, which would be expected to hyperpolarize the plasma membrane. When the $\Delta\Psi_{pm}$ was clamped with the transmembrane K^+ gradient by valinomycin addition, the rates of Put, Spd and TPP uptake were correlated to $[K^+]_o$ up to 100 mM KCl (Figure 2B) in a log-linear relationship fashion, as would be predicted by a Nernstian dependence of these parameters on $\Delta\Psi_{pm}$. These data suggest that Put and Spd transport are narrowly dependent on the K^+ diffusion potential as a result of its specific effect on $\Delta\Psi_{pm}$, and that the contribution of $\Delta\Psi_{mit}$ in driving [^{3}H]TPP uptake is responsible for the deviation observed between the rate of decrease of Put or Spd influx and of [^{3}H]TPP uptake when $[K^+]_o$ exceeds 20 mM.

Apparent lack of electrogenicity of diamine and polyamine transport

High rates of inward transport of diamines and polyamines would be expected to depolarize the plasma membrane if charge compensation is not stoichiometric during the carrier-mediated uptake of these compounds. We thus examined the effect of Put, Spd and Spm uptake on concomitant [^{3}H]TPP uptake at concentrations that nearly saturate their transport system(s). As

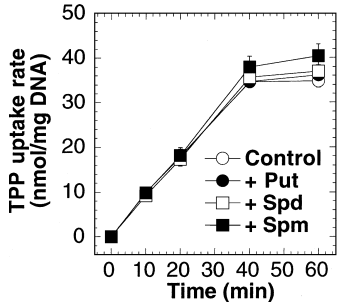


Figure 3 Effect of Put, Spd and Spm on [^{3}H]TPP uptake

ZR-75-1 cells were incubated in 400 μl of complete medium A supplemented with 20 μM Put (●), 5 μM Spd (□) or 5 μM Spm (■), or without amine supplementation (○) in the presence of 5 μM [^{3}H]TPP, and specific TPP uptake was determined at the indicated intervals. Values represent the means ± S.D. of triplicate determinations.

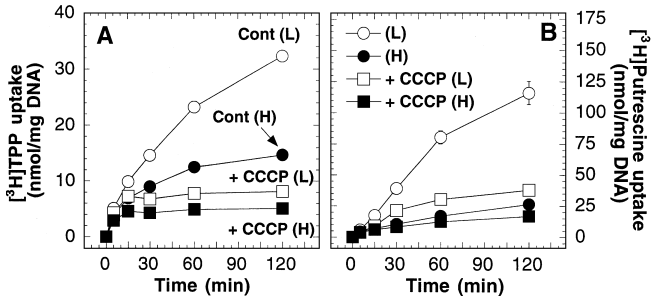


Figure 4 Effect of $[K^+]_o$ and CCCP on [^{3}H]TPP and [^{3}H]Put uptake in ZR-75-1 cells

At time zero, cells grown in standard growth medium were transferred to 400 μl of medium containing 1 μM CCCP (solid symbols) or the ethanol vehicle (0.01%, v/v) (Cont, plain symbols) and either 5.4 mM KCl (low $[K^+]_o$, L) (○, ●) or 100 μM KCl (high $[K^+]_o$, H) (□, ■), in the presence of either 5 μM [^{3}H]TPP (**A**) or 20 μM [^{3}H]Put (**B**). Other details are as described in the legend to Figure 1. Values represent the means ± S.D. of triplicate determinations from three independent experiments.

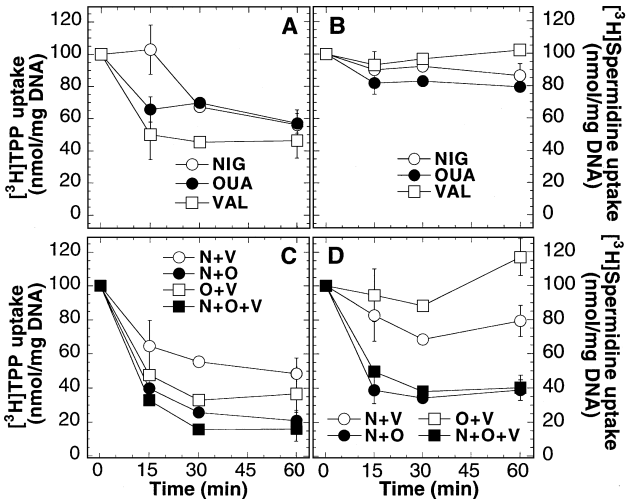


Figure 5 Effect of nigericin, ouabain and valinomycin on [^{3}H]Spd and [^{3}H]TPP uptake

At time zero, ZR-75-1 cells were transferred to 400 μl of complete medium A containing either 10 μM nigericin (N/G or N), 1 mM ouabain (OUA or O), 10 μM valinomycin (VAL or V), or the various combinations thereof, and either 5 μM [^{3}H]TPP (**A**, **B**) or 5 μM [^{3}H]Spd (**B**, **D**), and incubated for the indicated time interval before determination of specific [^{3}H]TPP or [^{3}H]Spd uptake. The EtOH concentration was kept constant in all experimental groups at 0.2% (v/v). (**A** and **B**) Cells treated with N (○), O (●) or V (□); (**C** and **D**) cells treated with N + V (○), N + O (●), O + V (□) or N + V + O (■). Values are expressed as percentages of specific uptake measured in control cells treated with the ethanol vehicle alone, and represent the means ± S.D. of triplicate determinations from three independent experiments.

shown in Figure 3, Put, Spd or Spm had no significant effect on [^{3}H]TPP uptake, indicating that diamine and polyamine uptake is apparently non-electrogenic, or produces changes in the $\Delta\Psi_{pm}$ that are too small to be detected with the method used.

Effect of protonophores and ouabain on Put and Spd transport

We performed several other manipulations of the $\Delta\Psi_{pm}$ and $\Delta\Psi_{mit}$ in order to corroborate the specific dependence of Put and Spd transport on $\Delta\Psi_{pm}$, and to rule out the possibility that $[K^+]_o$ was non-specifically inhibiting substrate internalization. As shown in Figure 4A, the addition of CCCP (10 μM), a rheogenic protonophore, decreased [^{3}H]TPP uptake at normal $[K^+]_o$ to a greater extent than valinomycin (cf. Figure 1A), suggesting that CCCP affected both $\Delta\Psi_{mit}$ and $\Delta\Psi_{pm}$. CCCP is expected to initially collapse $\Delta\Psi_{mit}$, soon followed by progressive depolarization of the plasma membrane as a result of net H^+ influx and ATP depletion [28,30,33]. Short-term ($\leqslant$ 4 h) exposure to CCCP at physiological pH does not affect cell viability [34], as reflected by the stability of residual [^{3}H]TPP accumulation throughout these experiments. As observed previously [11], CCCP depressed Put accumulation, albeit to a lower extent than exposure to 100 mM KCl (Figure 4B). Incubation with CCCP at high $[K^+]_o$ led to a further decrease in both [^{3}H]TPP and [^{3}H]Put accumulation, suggesting that plasma membrane depolarization by CCCP was only partial.

In order to change the $\Delta\Psi_{pm}$ without directly manipulating $[K^+]_o$, we determined the effect of ouabain and nigericin on Put and Spd transport. In the short term, ouabain is expected to decrease selectively the $\Delta\Psi_{pm}$ by dissipating the component due the electrogenic activity of the Na^+/K^+-ATPase [35]. On the other hand, nigericin, a non-rheogenic K^+/H^+ ionophore [26], may hyperpolarize mitochondria by dissipating the pH gradient, leading to a compensatory increase in the electrical component of the $\Delta\Psi_{mit}$. A secondary plasma-membrane hyperpolarization is also sometimes observed in epithelial cells treated with nigericin [27,28]. As shown in Figure 5A, ouabain selectively depolarized the plasma membrane, as evidenced by the additive inhibition of [^{3}H]TPP uptake by valinomycin and ouabain (Figure 5C). However, the Na^+/K^+-ATPase inhibitor only weakly decreased Spd uptake (Figure 5B), although Put transport was slightly more sensitive to ouabain (cf. Table 1) [11]. Nigericin induced a transitory increase in [^{3}H]TPP uptake within 10 min after its addition (results not shown), but more prolonged incubation decreased intracellular accumulation of the potential probe (Figure 5A), while having little effect on [^{3}H]Spd (Figure 5B). On the other hand, nigericin depressed Spd uptake by 60% (Figure 5D) in the presence of ouabain, but not valinomycin. The markedly synergistic inhibition of Spd transport induced by co-incubation with nigericin and ouabain was accompanied by a dramatic drop in [^{3}H]TPP accumulation (Figure 5C). As expected, valinomycin reduced [^{3}H]TPP uptake as a result of mitochondrial membrane depolarization in cells treated with either nigericin or ouabain alone (Figure 5C). The fact that further addition of valinomycin caused only marginal reduction in [^{3}H]TPP uptake in ZR-75-1 cells co-incubated with nigericin

Table 1 Effect of extracellular Na^+ on the inhibition of putrescine uptake by nigericin and ouabain

ZR-75-1 cells were preincubated for 10 min in a CO_2-free incubator in medium A^- containing either 103 mM NaCl as the sole source of Na^+ ($+Na^+$), or 200 mM sucrose ($-Na^+$) (see the Materials and methods section). Cells were then incubated for 20 min in the presence of the indicated compounds and 20 μM [^{3}H]Put for the determination of uptake activity. Values are the means $\pm$ S.D. of triplicate determinations; values between parentheses are expressed as percentages of the respective, untreated control cells. Where indicated, values are significantly different from those of untreated cells (* $P < 0.01$, ** $P < 0.05$).

Treatment	[^{3}H]Put uptake			
	$+Na^+$ (nmol/30 min per mg of DNA)	(% of control)	$-Na^+$ (nmol/30 min per mg of DNA)	(% of control)
Control	32.1 $\pm$ 4.5	100	47.7 $\pm$ 1.5	100
+10 μM nigericin	28.0 $\pm$ 1.2	87	64.6 $\pm$ 3.2*	135
+1 mM ouabain	19.8 $\pm$ 2.8*	62	38.2 $\pm$ 1.8*	80
+10 μM nigericin +1 mM ouabain	0.7 $\pm$ 0.4*	2	35.3 $\pm$ 1.4**	74

and ouabain is consistent with a very rapid plasma-membrane depolarization, thus preventing mitochondrial accumulation of the probe [27]. Quite notably, Spd uptake inhibition by either nigericin or ouabain was not enhanced by simultaneous addition of valinomycin (Figure 5D), pointing to a specific effect of nigericin on $\Delta\Psi_{pm}$.

In mammalian cells, cytoplasmic acidification caused by nigericin-induced H^+ influx is rapidly counteracted by intracellular pH (pH_i) homoeostatic mechanisms, including the activation of Na^+/H^+ and HCO_3^-/Cl^- antiporters [36,37]. Thus, blocking Na^+/K^+-ATPase activity could dissipate the Na^+ electrochemical gradient required to maintain Na^+/H^+ antiporter activity, and thus exacerbate nigericin-induced acidification. If decreased pH_i was responsible for Put- and Spd-uptake inhibition, this would predict that amiloride analogues with high specificity for Na^+/H^+ antiporter inhibition would depress these transport activities in a nigericin-dependent manner. However, as described below, amiloride and its analogues strongly inhibited Put and Spd transport in the absence of the ionophore. On the other hand, nigericin transiently decreased pH_i by ≈ 0.2 in ZR-75-1 cells under the experimental conditions used, as spectrofluorometrically determined in cells pre-loaded with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (C. Zhao and R. Poulin, unpublished work). Full pH_i recovery was observed within 10 min after protonophore addition, and ouabain did not retard this regulatory pH_i increase as expected, since Na^+/H^+ activity is dispensable for pH_i homeostasis in HCO_3^--containing media at physiological external pH [36,38].

Since nigericin also expels intracellular K^+ at a high rate, and because H^+ influx is either buffered in the cytoplasm, or converted into net Na^+ influx through Na^+/H^+ exchange, Na^+/K^+-ATPase activity may be required to preserve both the K^+ diffusion potential and the $\Delta\Psi_{pm}$ against nigericin action. Thus Put and Spd uptake could be synergistically inhibited by nigericin and ouabain through the rapid dissipation of the $\Delta\Psi_{pm}$ at the expense of a reversal of the normal Na^+ and K^+ electrochemical gradients. This would predict that the potentiation of Put uptake inhibition by nigericin and ouabain would be strongly Na^+-dependent. Indeed, in a Na^+-, HCO_3^--free medium, nigericin alone strongly increased Put transport, whereas the further addition of ouabain prevented this stimulation while maintaining Put uptake activity to near control levels (Table 1). This markedly contrasts with the virtual suppression of Put transport by co-incubation with nigericin and ouabain observed in Na^+-replete medium. As observed previously [11], complete iso-osmotic replacement of Na^+ with sucrose in the uptake medium increased Put transport, in agreement with the observation that this process is Na^+-independent, and that in fact Na^+ is a weak competitive inhibitor of Put uptake. Selective substitution of NaCl with the chloride salts of Li^+, choline or *N*-methyl-D-glucamine was not feasible, since these cations lead to a strong inhibition of Put transport [11]. Nigericin strongly increased [^{3}H]TPP uptake in Na^+-free medium, and this effect was prevented by ouabain (results not shown), clearly suggesting that nigericin hyperpolarizes the plasma membrane, and that this tendency is counteracted by the presence of extracellular Na^+. The decrease in ionic strength resulting from iso-osmotic substitution of NaCl with sucrose had no effect on basal $\Delta\Psi_{pm}$, indicating that the differential effect of nigericin in this system was largely independent from changes in the ionic composition of the medium. This is consistent with the fact that the $\Delta\Psi_{pm}$ is mainly determined by the transmembrane K^+ gradient in epithelial cells [26,27], which was kept intact in these experiments.

Role of $\Delta\Psi_{pm}$ in Put efflux

Since an electrodiffusive pathway of Spd efflux has been recently identified in *Xenopus* oocytes [25], we assessed whether Put export was also controlled by the $\Delta\Psi_{pm}$. We took advantage of the fact that the steady-state Put pool in ZR-75-1 cells is extremely small [9], and thus, the specific [^{3}H]Put radioactivity should be comparable between the intra- and extra-cellular compartments for short incubation periods. For this purpose, ZR-75-1 cells were pre-loaded with [^{3}H]Put for 20 min, and, after washing, cell monolayers were incubated in Put-free medium in the presence of various ionophores (Figure 6B) or at high $[K^+]_o$ (Figure 6A) to manipulate the $\Delta\Psi_{pm}$. For comparison, the effects of moderate hyperosmotic stress imposed by NaCl addition, as well as the exchangeability of internalized [^{3}H]Put by the addition of 500 μM unlabelled Put, were studied in parallel (Figure 6A). We have previously determined that under these experimental conditions, most of the accumulated Put is not metabolized [9]. None of these treatments significantly ($P > 0.5$) affected the rapid initial release of internalized [^{3}H]Put observed within 5 min after substitution with label-free medium. Hyperosmotic stress and incubation with unlabelled Put slowly increased release of internalized substrate (by up to 50 %) over a 2 h period. On the other hand, depolarization with either CCCP or high $[K^+]_o$ had no significant effect ($P > 0.5$) on net Put efflux, whereas nigericin almost completely blocked further [^{3}H]Put release after the initial rapid excretion noted at 5 min. Valinomycin also significantly decreased net Put efflux upon prolonged incubation (60–120 min). Although significant salvaging or reuptake of excreted [^{3}H]Put could have occurred during the 2 h experimental period, this does not invalidate the observation that net Put excretion was unaffected by agents such as CCCP or high $[K^+]_o$

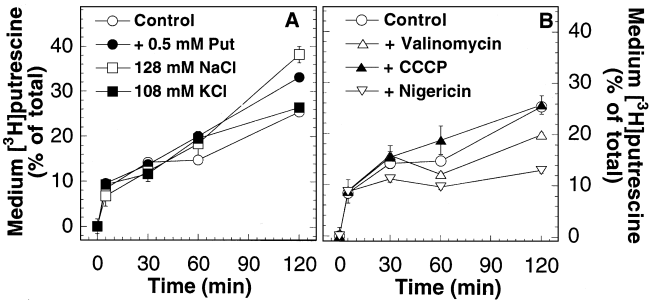


Figure 6 Effect of extracellular Na^+, K^+ and ionophores on net [^{3}H]Put efflux

ZR-75-1 cells were pre-incubated for 20 min with 20 μM [^{3}H]Put in 400 μl of RPMI 1640 before incubation for the indicated time interval in 1 ml of label-free medium and determination of extracellular radioactivity, as described in the Materials and methods section. (**A**) Cells were incubated in complete medium A alone (control, ○), or supplemented with either 0.5 mM unlabelled Put (●) or 25 mM NaCl (total NaCl concentration = 128 mM) (□); cells were also incubated in medium A$^-$ containing 108 mM KCl (■) (see the Materials and methods section). (**B**) Cells were incubated in complete medium A alone (○), or containing 10 μM valinomycin (△), 1 μM CCCP (▲), or 10 μM nigericin (▽). Values are expressed as the percentages of total radioactivity recovered in the medium per cell culture, and represent the means ± S.D. from triplicate determinations.

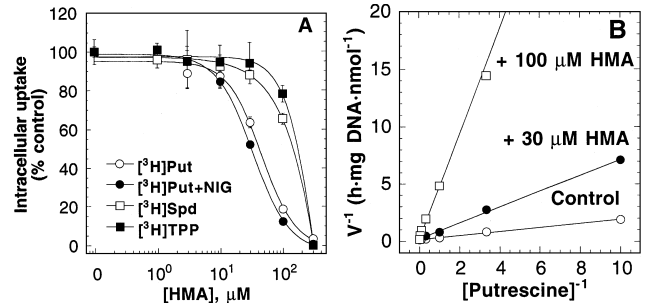


Figure 7 Effect of HMA on [^{3}H]Put, [^{3}H]Spd and [^{3}H]TPP uptake

(**A**) Cells were incubated for 20 min in RPMI 1640 medium containing 20 μM [^{3}H]Put alone (○) or in the simultaneous presence of 10 μM nigericin (NIG) (●), 5 μM [^{3}H]Spd (□), or 5 μM [^{3}H]TPP (■), and increasing concentrations of HMA. Specific uptake of the radiolabelled substrates was determined at the end of the experimental period. Values represent the means ± S.D. of triplicate determinations from two independent experiments. (**B**) Lineweaver–Burk analysis of the inhibition of [^{3}H]Put uptake by HMA. (○) Control; (●) +30 μM HMA; (□) +100 μM HMA.

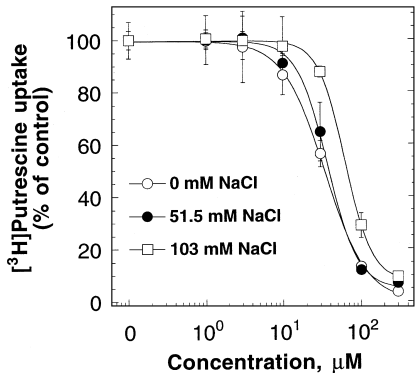


Figure 8 Effect of extracellular Na^+ on HMA-induced inhibition of Put uptake

ZR-75-1 cells were incubated for 20 min in a CO_2-free incubator in the presence of 20 μM [^{3}H]Put and 0 (○), 51.5 (●), or 103 mM NaCl (□). Osmolality was kept constant by sucrose addition, as described in the Materials and methods section. Specific Put uptake was determined at the end of the experimental period. Values are expressed as percentages of Put uptake measured in the absence of HMA, and represent the means ± S.D. of three independent experiments.

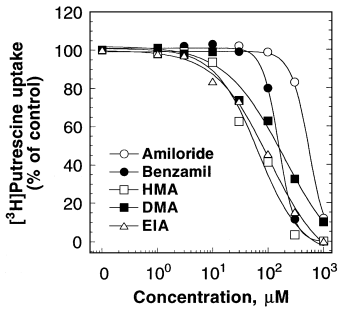


Figure 9 Inhibition of [^{3}H]Put uptake by various amiloride analogues

Cells were incubated for 20 min in RPMI 1640 medium containing 20 μM [^{3}H]Put alone and increasing concentrations of amiloride (○), benzamil (●), HMA (□), 5-*N*,*N*-dimethylamiloride (DMA, ■) or 5-(*N*-ethyl,*N*-isopropyl)amiloride (EIA, △). Specific uptake of the radiolabelled substrates was determined at the end of the experimental period. Values are expressed as percentages of Put uptake in the absence of HMA, and represent the means ± S.D. of three independent experiments.

at early time points, since these treatments strongly inhibit influx of the diamine.

Demonstration of a high-affinity amiloride-binding site on the Put carrier

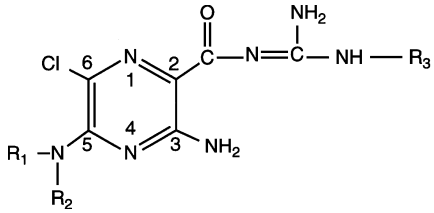
Plasma-membrane transport of organic cations such as tetraethylammonium or *N*-methylnicotinamide, is thought to be catalysed in epithelial mammalian cells through an organic cation/H^+ exchange system energized by the pH gradient generated through the activity of the Na^+/H^+ antiporter [39]. In order to assess whether a similar coupling could provide the driving force for Put and Spd uptake, we examined the effect of HMA, a potent inhibitor of the mammalian Na^+/H^+ antiporter [40], on the inward uptake of these substrates. Surprisingly, as shown in Figure 7, HMA completely inhibited Put uptake in a concentration-dependent manner with an IC_{50} 66 ± 17 μM. Simultaneous addition of nigericin, which would be expected to stimulate Na^+/H^+ exchange, did not notably potentiate HMA-induced inhibition of Put transport. Spd transport was also inhibited by HMA, but only at concentrations (> 100 μM) which also abolished net [^{3}H]TPP uptake. This effect was observed immediately (< 2 min) after addition of HMA and was rapidly reversible (results not shown). Kinetic analysis clearly showed that HMA inhibits Put transport through a competitive mechanism, with a calculated K_i of 4.0 ± 0.9 μM (Figure 7B), which is comparable with the affinity for putrescine uptake (K_m 3.7 μM) in ZR-75-1 cells [9]. This value is 2–3 orders of magnitude larger than that observed for the inhibition of the mammalian Na^+/H^+ exchanger by HMA, even in Na^+-containing media [41].

Since amiloride and its analogues specifically interact with Na^+-binding sites of membrane proteins [40,42], we assessed the possibility that inhibition of Put uptake by HMA might be enhanced by decreasing the extracellular Na^+ concentration. The IC_{50} values of Put uptake inhibition by HMA measured at 0, 51.5

Table 2 Relative potency of amiloride analogues to inhibit the putrescine carrier, the NHE-1 isoform of the Na^+/H^+ antiporter, diamine oxidase activity and Na^+/D-glucose co-transporter activity

IC_{50} values (±S.D.) for putrescine-uptake inhibition are averaged from results from Figure 9 and similar experiments. Relative potencies for inhibition of the following proteins are from literature data: the NHE-1 Na^+/H^+ antiporter [40,48], human kidney diamine oxidase (DAO) [47], and the rabbit kidney Na^+/D-glucose transporter (SGLT-1) [49]. The chemical groups refer to the general amiloride structure given below. Abbreviations used: DMA, 5-*N*,*N*-dimethylamiloride; EIA, 5-(*N*-ethyl,*N*-isopropyl)amiloride.

				Put uptake		Relative potency (benzamil = 1)		
Compound	R_1	R_2	R_3	IC_{50} (μM)	Relative potency (benzamil = 1)	NHE-1	DAO	SGLT-1
Amiloride	H	H	H	> 500	< 0.3	25	0.1	0.25–0.5
Benzamil	H	H	benzyl	155±5	1	1	1	1
DMA	CH_3	CH_3	H	189±17	0.82	5×10^2	–	1
EIA	CH_2CH_3	$CH(CH_3)_2$	H	104±20	1.5	5×10^3	0.5	–
HMA	-hexamethylene-		H	66±17	2.3	1×10^4	–	–



and 103 mM NaCl were 33±2, 37±3 and 63±2 μM (Figure 8). The 2-fold potentiation of Put uptake inhibition by HMA observed upon deletion of extracellular Na^+ is consistent with the fact that the apparent K_m for Put transport under those conditions is shifted from 3.7 to 1.5 μM due to competitive inhibition by Na^+ with a $K_i \approx 139$ mM [11]. The ability of a series of amiloride analogues to inhibit Put uptake was also determined to obtain a pharmacological profile of Put transport inhibition. As shown in Figure 9 and Table 2, the order of potency of amiloride analogues to inhibit Put transport was HMA > 5-*N*-ethyl,5-*N*-isopropyl-amiloride > 5-*N*,*N*-dimethylamiloride ≈ benzamil > amiloride.

DISCUSSION

The present results clearly demonstrate that Put and Spd influx is dependent on $[K^+]_o$ as a result of its narrow control by $\Delta\Psi_{pm}$, and not from mere competition by K^+ for binding to the respective carrier(s). As predicted from a Nernstian dependence of the transport activities on the K^+ diffusion potential, clamping the K^+ transmembrane gradient with valinomycin led to a log-linear relationship, rather than Michaelis–Menten-type kinetics of inhibition, between $[K^+]_o$ and Put, Spd and TPP uptake within a large range of $[K^+]_o$, including both sub- and supra-physiological concentrations. Moreover, the present data clearly show that the rate of intracellular Put and Spd uptake is independent of $\Delta\Psi_{mit}$. If $\Delta\Psi_{mit}$ is the main driving force for mitochondrial polyamine uptake [29], these results indicate that mitochondrial compartmentalization of Put and Spd is negligible in short-term incubations, and that these compounds do not behave as lipophilic cations like TPP, despite their amphiphilic character.

The fact that CCCP, unlike valinomycin, strongly and rapidly inhibited Put and Spd uptake suggests that the protonophore elicits uncompensated H^+ influx that counteracts their inward transport. Although CCCP induces slight, transient intracellular acidification even in the absence of a transmembrane pH gradient, this fall in pH_i subsides within minutes through the activation of pH_i homoeostatic mechanisms such as Na^+/H^+ antiporter activity [34]. A decrease in pH_i is thus unlikely to contribute significantly to the inhibition of Put or Spd uptake by CCCP. Since the decrease in [^{3}H]TPP uptake elicited by high $[K^+]_o$ in the presence of CCCP was only marginal, the main effect of CCCP on Put and Spd transport most likely results from plasma-membrane depolarization, inasmuch as pH_i homoeostatic mechanisms are electroneutral [37], and, thus, cannot compensate the fall in $\Delta\Psi_{pm}$ due to the net influx of positive charges. This is further supported by the finding that, in the presence of CCCP, valinomycin increases Put and Spd uptake in ZR-75-1 cells [11]. This phenomenon most likely results from the tendency of valinomycin to hyperpolarize slightly the plasma membrane, an effect that becomes more evident at lower $\Delta\Psi_{pm}$ [33].

The inability of nigericin to inhibit substantially Put and Spd uptake, as compared with CCCP, is consistent with the non-rheogenic character of this K^+/H^+ ionophore. As discussed above for CCCP, intracellular acidification by nigericin is unlikely to inhibit *per se* Put uptake, since the ionophore in fact markedly elevated Put transport under conditions (Na^+-HCO_3^--free medium) where pH_i homoeostasis should be strongly impaired. Interestingly, simultaneous addition of ouabain and nigericin completely abolished Put uptake and markedly inhibited Spd transport, with strong concomitant plasma-membrane depolarization. Na^+-dependent transport processes were clearly involved in the suppression of Put uptake by co-treatment with nigericin and ouabain. Moreover, nigericin strongly hyperpolarized ZR-75-1 cells incubated under Na^+-, HCO_3^--free, but not under Na^+-replete, conditions, with concomitant activation of Put transport. The hyperpolarizing effect of nigericin on the $\Delta\Psi_{mit}$ is known to induce a secondary, ouabain-sensitive hyperpolarization of the plasma membrane in some epithelial cells, although the mechanism is not well understood [27,28]. Our data strongly suggest that, in ZR-75-1 cells, Na^+-dependent transport mechanisms

tend to offset this nigericin-dependent hyperpolarization, and that Na^+/K^+-ATPase activity is required for this elevation of the $\Delta\Psi_{pm}$. Therefore, the synergistic effect of nigericin and ouabain on $\Delta\Psi_{pm}$, and on Put and Spd uptake, can be best attributed to the dual role of the Na^+/K^+-ATPase in preventing dissipation of both the transmembrane K^+ gradient by nigericin [43], and of the electrochemical Na^+ gradient by Na^+-influx pathways, including the Na^+/H^+ antiporter.

Net Put efflux in ZR-75-1 cells was not enhanced by drops in $\Delta\Psi_{pm}$ induced by CCCP or high $[K^+]_o$, but was significantly inhibited by slightly hyperpolarizing stimuli such as nigericin and valinomycin. Thus, in ZR-75-1 cells, Put efflux is apparently sensitive to a different, more negative, range of $\Delta\Psi_{pm}$ values. This behaviour would suggest that Put uptake is essentially unidirectional, and that plasma-membrane depolarization does not lead to a rapid efflux of accumulated substrate. A similar phenomenon has previously been noted in *E. coli*, and might reflect sequestration of internalized Put by intracellular binding sites [24]. The lack of Put efflux in response to plasma-membrane depolarization in ZR-75-1 cells might be accounted for by the fact that the electrodiffusive efflux pathway, such as that described in *Xenopus* oocytes, is inhibited by divalent cations present in the medium used in our assays [25]. Taken together, these findings support the notion that the $\Delta\Psi_{pm}$-dependent pathway of Put uptake is unidirectional and clearly different from the export system described in mammalian cells [25,44]. Thus Put and Spd transport is catalysed by a saturable, Na^+-independent and apparently unidirectional carrier system that is mostly driven by the $\Delta\Psi_{pm}$. These characteristics are similar to those found for the system y^+ basic amino acid transporter [45] (e.g. mBAT-1), except that the latter is bi-directional. It is as yet unknown whether Put or Spd transport is electrogenic, but transfer of positive charges must be rapidly compensated, as evidenced from the lack of effect of Put and Spd influx on $\Delta\Psi_{pm}$.

The possibility that Na^+/H^+ antiporter activity provides a hypothetical pH gradient essential for Put and Spd transport, or is required to prevent acidification by nigericin, was explored by using HMA, a potent inhibitor of this exchanger. Unexpectedly, HMA, as well as amiloride and a number of its analogues, were found potently to inhibit Put and, to a lesser degree, Spd transport. HMA clearly inhibited Put transport in a competitive fashion, strongly suggesting a direct interaction with the Put carrier. HMA also abruptly abolished net [^{3}H]TPP uptake at concentrations $> 100\ \mu$M, but the contribution of this effect to Put-uptake inhibition was minimal. The weaker Spd-uptake inhibition by HMA could reflect the $\approx$ 6-fold higher affinity of Spd for its specific uptake in ZR-75-1 cells as compared with Put [9], and its specificity is uncertain, since [^{3}H]TPP uptake was inhibited with a comparable concentration-dependence. The abrupt suppression of [^{3}H]TPP uptake by HMA has not been previously reported to our knowledge. Although amiloride and HMA induce a slow, partial depolarization of the plasma membrane in some tissues [41], inhibition of [^{3}H]TPP uptake by 300 μM HMA was complete, immediate and rapidly reversed after removing the analogue. Thus suppression of [^{3}H]TPP uptake by high HMA concentrations could result from competition with [^{3}H]TPP for binding to the plasma membrane, inasmuch as interaction of hydrophobic ions such as TPP with pure lipid bilayers exhibits saturation in the 10^{-4} M range, presumably due to the electrostatic component of this interaction [46].

The present results provide original evidence that the Put transporter might be an amiloride-binding protein. In addition to the Na^+/H^+ antiporter, amiloride and its analogues bind to topologically similar regions of several ion transport proteins, such as the Na^+/phosphate and Na^+/D-glucose co-transporters and the Na^+ epithelial channel [42]. It is noteworthy that human kidney diamine oxidase, a Cu^{2+}-dependent enzyme with a 2,4,5-trihydroxyphenylalanine prosthetic group that oxidatively deaminates diamines, was originally identified as an amiloride-binding protein [47]. As for Put transport, amiloride and its analogues competitively inhibit diamine oxidase activity, but guanidino-substituted analogues such as benzamil are more potent than amiloride and 5-substituted analogues, in contrast with the higher potency of the latter class of analogues to inhibit Put uptake (cf. Table 2) [47]. The relative inhibitory potency of the various analogues observed toward Put uptake was also clearly different from that reported for the ubiquitously expressed NHE-1 isoform of the mammalian Na^+/H^+ antiporter, since guanidino-substituted compounds such as benzamil are weaker inhibitors than amiloride towards this exchanger [48]. In fact, Put-uptake inhibition by amiloride analogues had pharmacological characteristics somewhat similar to those found for Na^+-cotransport proteins such as the Na^+/D-glucose facilitator SGLT1 [49], which is more sensitive to benzamil and 5-substituted amiloride analogues than to amiloride itself (Table 2). Although Put transport is clearly Na^+-independent, Na^+ is a weak competitive inhibitor of Put uptake [11]. The present data show that the decrease in the IC_{50} value for Put-uptake inhibition by HMA upon transfer to Na^+-free medium was quantitatively similar to the apparent increase in Put affinity for transport noted under the same conditions [11]. We cannot rule out the possibility that the reduction in ionic strength and Cl^- due to iso-osmotic substitution of NaCl with sucrose was partly responsible for the increased potency of Put-transport inhibition by HMA, since replacing NaCl with the chloride salts of Li^+, choline, or *N*-methyl-D-glucamine could not be performed due to their strong inhibitory effect on Put uptake [11,13,15]. Nevertheless, it is clear that the efficiency of the interaction between HMA and the Put-transport system is influenced by the ionic composition of the medium in a manner quantitatively and qualitatively similar to the affinity of the Put carrier for its substrate, thus providing strong support for the competitive mode of inhibition of Put transport by 5-alkylated amiloride analogues. Since amiloride-binding domains often coincide with Na^+-binding sites [42], the present data suggest that the substrate-binding site of the Put carrier might also be a high-affinity amiloride-binding site. The relationship of the Put carrier to other amiloride-binding cation transporters should be elucidated with the molecular identification of this membrane protein.

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