Polyamine uptake by the intra-erythrocytic malaria parasite, *Plasmodium falciparum*

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Running title: Polyamine transport in the malaria parasite

Abstract

Polyamines and the enzymes involved in their biosynthesis are present at high levels in rapidly proliferating cells, including cancer cells and protozoan parasites. Inhibition of polyamine biosynthesis in asexual blood-stage malaria parasites causes cytostatic arrest of parasite development under *in vitro* conditions, but does not cure infections *in vivo*. This may be due to replenishment of the parasite's intracellular polyamine pool via salvage of exogenous polyamines from the host. However the mechanism(s) of polyamine uptake by the intra-erythrocytic parasite are not well understood. In this study, the uptake of the polyamines putrescine and spermidine into *Plasmodium falciparum* parasites functionally isolated from their host erythrocyte were investigated using radioisotope flux techniques. Both putrescine and spermidine were taken up into isolated parasites via a temperature-dependent process that showed cross-competition between different polyamines. There was also some inhibition of polyamine uptake by basic amino acids. Inhibition of polyamine biosynthesis led to an increase in the total amount of putrescine and spermidine taken up from the extracellular medium. The uptake of putrescine and spermidine by isolated parasites was independent of extracellular Na^+ but increased with increasing external pH. Uptake also showed a marked dependence on the parasite's membrane potential, decreasing with membrane depolarization and increasing with membrane hyperpolarization. The data are consistent with polyamines being taken up into the parasite via an electrogenic uptake process, energized by the parasite's inwardly negative membrane potential.

Keywords: Malaria, *Plasmodium*, polyamine transport, spermidine, putrescine

1. Introduction

Polyamines are aliphatic, low-molecular weight nitrogenous bases consisting of methylene moieties separating two to four amine groups, which are protonated at physiological pH (Wallace et al., 2003). These compounds, together with the enzymes involved in their biosynthesis, are present at high levels in rapidly proliferating cells, including cancer cells and protozoan parasites (Birkholtz et al., 2011; Casero and Woster, 2009). The physiological roles of polyamines are not completely understood (Jänne et al., 2004). Nevertheless, there is significant interest in targeting polyamine synthesis as a basis for chemotherapy; e.g. the polyamine biosynthesis inhibitor α-difluoromethylornithine (DFMO, Ornidyl®) is used for the treatment of West African sleeping sickness caused by the parasite *Trypanosoma brucei gambiense* (Bacchi et al., 1990), and DFMO is also under investigation as an anticancer agent (Casero and Woster, 2009).

The asexual intraerythrocytic form of the human malaria parasite, *Plasmodium falciparum*, has a high internal concentration of polyamines (Assaraf et al., 1984; Teng et al., 2009). The biosynthesis of polyamines by this parasite has some unusual features (Müller et al., 2000). In particular, two of the enzyme activities involved, *S*-adenosylmethionine decarboxylase (AdoMetDC) and ornithine decarboxylase (ODC) reside within a bifunctional protein, encoded by a single open reading frame. This bifunctional arrangement of AdoMetDC and ODC is unique to Plasmodia and is postulated to be necessary for the regulation of polyamine production (Williams et al., 2011). Under *in vitro* conditions, inhibition of this enzyme by compounds such as DFMO results in growth arrest of the intra-erythrocytic *P. falciparum* parasite. This arrest can be overcome by the addition of exogenous polyamines (Assaraf et al., 1987b; Das Gupta et al., 2005; Wright et al., 1991), consistent with the presence in the parasite of polyamine uptake mechanism(s). Disruption of polyamine metabolism as a basis for antimalarial chemotherapy may therefore require the inhibition of both polyamine biosynthesis and the uptake of exogenous polyamines.

Previous studies have investigated the transport of the diamine putrescine into rhesus monkey erythrocytes infected with the primate parasite *P. knowlesi* (Singh et al., 1997), and into *P. falciparum* infected human erythrocytes (Ramya et al., 2006). However, the mechanism by which polyamines gain entry into the intraerythrocytic parasite has not been described, and nor is there an obvious polyamine-specific transporter encoded in the *P. falciparum* genome (Martin et al., 2005). In this study, we investigated the uptake of the diamine, putrescine, and the triamine, spermidine, into parasites functionally isolated from their host erythrocytes by saponin-permeabilization of the host cell membrane.

2. Materials and methods

2.1 Solutions, cell culture and cell preparation

A range of different HEPES-buffered salines was used in this study: solution A (125 mM NaCl, 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution B (130 mM NaCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution C (130 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution D (135 mM NMDG [N-methyl-D-glucamine], 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); and solution E (125 mM NaCl, 5 mM KCl, 20 mM glucose,15 mM HEPES, 10 mM MES [2-morpholinoethanesulfonic acid] and 1 mM $MgCl_2$, pH 6.1, 7.1, 8.1). For competition experiments, solutions of the compounds of interest (e.g. amino acids, polyamines) were prepared as 100 mM stocks in Solution A.

P. falciparum infected erythrocytes (strain 3D7) were cultured under shaking conditions as described elsewhere (Allen and Kirk, 2010) and synchronized by lysing mature trophozoite-stage parasitized erythrocytes by suspension of the cultured cells in an iso-osmotic sorbitol solution (Lambros and Vanderberg, 1979). Experiments were performed with mature trophozoite-stage (30-36 h post-invasion) *P. falciparum* parasites that had been functionally isolated from their host blood cell by saponin-permeabilization of the erythrocyte and parasitophorous vacuole membranes using 0.05% w/v saponin, as described elsewhere (Saliba et al., 1998; Spillman et al., 2008). Following the saponin-treatment, the isolated parasites were resuspended in solution A for 10 min at 37°C before being washed three times with the solution required for each experiment, then resuspended to a final concentration of 0.5-1x10^8 cells/ml as estimated using an improved Neubauer counting chamber.

2.2. Uptake of polyamines into isolated parasites

The uptake of [^{3}H]putrescine or [^{3}H]spermidine into saponin-isolated *P. falciparum* parasites was initiated by combining solution A-E (as appropriate), containing either [^{3}H]putrescine or [^{3}H]spermidine, with an equal volume of cell suspension, giving a final cell concentration of 0.5-

$1x10^8$ cells/ml. In some experiments, the radiolabelled polyamines were present at sufficient concentration to give a final concentration of radioactivity of 0.1 µCi/ml (equating to final concentrations of putrescine and spermidine of 5 nM and 6 nM, respectively); in others the concentrations of the radiolabelled polyamines were increased five-fold.

In the majority of experiments, the uptake of radiolabel was terminated by centrifuging the parasites through an oil layer. At predetermined time intervals (time courses) or, in some experiments, after a single time-period, 200 µl aliquots of the cell suspension (in triplicate for [^{3}H]putrescine uptake, quadruplicate for [^{3}H]spermidine uptake) were transferred to microcentrifuge tubes containing a dibutyl/dioctyl phthalate oil blend (5:4; 1.015 g/ml). The tubes were immediately centrifuged (17 000 x g for 1 min), sedimenting the cells below the oil layer, thereby terminating uptake. A 10 µl sample of the aqueous phase above the oil layer was transferred to a scintillation vial to allow an estimate of the extracellular radiolabel concentration The aqueous phase was then aspirated from above the oil layer, and the tube and oil layer were rinsed three times with water to remove residual radioactivity before aspirating the oil. In initial experiments a small volume (30 µl) of 30% (v/v) perchloric acid was included beneath the dibutyl/dioctyl phthalate layered so that on centrifugation of the isolated parasites beneath the oil the cells were lysed and the associated proteins precipitated. In later experiments the perchloric acid beneath the oil was omitted. In the former experiments the aspiration of the oil was followed by the addition of 5% w/v trichloroacetic acid (1 ml) to each sample (Martin and Kirk, 2007). In the latter experiments, following the aspiration of the oil the cell pellets were lysed with 0.1% (v/v) Triton X-100 (0.5 ml) and the proteins precipitated with 5% w/v trichloroacetic acid (0.5 ml). In both cases the samples were centrifuged at 17 000 x g for 10 min to clear the cellular debris before measuring the radioactivity present in the supernatant using a β-scintillation counter.

In a number of the single time-period experiments (e.g. those investigating the membrane potential dependence of [^{3}H]putrescine uptake) the parasites were separated from the extracellular solution using an alternative approach (not involving an oil layer). Aliquots (600 µl) of the (radiolabel-containing) suspension were transferred to microcentrifuge tubes which were then centrifuged at 8

000 x g for 1 min to sediment the cells. The supernatant solution was immediately aspirated and the cells resuspended in an ice-cold aliquot (1 ml) of the solution being used for each experiment (excluding the radiolabel) before sedimenting the cells at 17 000 x g for 1 min. The supernatant solution was aspirated and the cell pellet lysed and processed as described above. All uptake measurements were made at 37°C, except where stated otherwise.

Polyamines carry multiple positive charges at physiological pH, and these can lead to electrostatic interactions with the negatively charged components of membranes (Schuber, 1989). This complicates polyamine uptake studies since a significant proportion of the cell-associated polyamines may be adhering to the cell surface (Pistocchi et al., 1988). In *Escherichia coli,* it was shown that this absorbed component increased with increasing valency of the polyamine (Tabor and Tabor, 1966). In each experiment of the present study the amount of radiolabeled polyamine on the outer surface of the cells, together with that trapped in the extracellular space of the cell pellet, was estimated either by extrapolating time-course data to 'time-zero', or by taking replicate samples as quickly as possible after combining the cells and radiolabel and immediately terminating the uptake of radiolabel. This amount was then subtracted from the total amount of radioactivity associated with the cell pellet to give an estimate the amount of radiolabel taken up into the parasites. In a typical [^{3}H]putrescine uptake experiment the *extracellular* radioactivity in the cell pellet (i.e. that associated with the cell surface and trapped in the extracellular solution) was of the order of 60% of the total radioactivity in the pellet following equilibration of the radiolabel between the intra- and extracellular solutions. In the case of [^{3}H]spermidine the extracellular radioactivity in the cell pellet was of the order of 75% of the total radioactivity taken up following equilibration of the radiolabel.

For the purpose of most of the figures the uptake of radiolabelled polyamines is represented as a 'distribution ratio'; i.e. the apparent concentration of radiolabelled polyamine inside the cell relative to that in the extracellular solution. The apparent concentration of radiolabel inside the cell was estimated by dividing the amount of radiolabel taken up into the parasites by the number of the

cells in the pellet and by the intracellular water volume of an individual isolated parasite (estimated previously as 28 fl; Saliba et al., 1998).

2.3 Measurement of the cytosolic pH of isolated P. falciparum *parasites*

The effect of putrescine and spermidine import on the cytosolic pH (pH_i) was measured by preloading isolated parasites suspended in either Solution A or Solution E with the fluorescent pH-sensitive dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), and monitoring the fluorescence with a spectrofluorometer as described elsewhere (Saliba and Kirk, 1999).

2.4 Data analysis

Unless specified otherwise, the data presented represent the means from at least three independent experiments, ± the standard-error-of-the-mean (SE). The statistical significance of any differences observed was determined either by the two-tailed t-test using the Graphpad Instat (v 3.06) program, or by the Wilcoxon Matched Pairs Test using Statistica (v9). Non-linear regression was performed with SigmaPlot (v.11). Initial rates of putrescine or spermidine uptake were calculated from uptake time-course data by fitting the data to the first order equation $y = a \times (1-e^{-kt})$, where y is the amount (pmol/10^{10} cells) of putrescine or spermidine imported, t is the time, *a* is the maximum amount of putrescine or spermidine imported and *k* is the first order rate constant. The product of *a* and *k* gives the initial rate of [^{3}H]putrescine or [^{3}H]spermidine uptake. For kinetic measurements, the K_m and V_{max} were determined by fitting the data to the Michaelis-Menten equation where $V_0 = V_{max}[polyamine]/(K_m + [polyamine])$ with V_0 the initial rate of putrescine or spermidine uptake, V_{max} the maximum initial uptake rate for putrescine or spermidine, [polyamine] the concentration of putrescine or spermidine and K_m the putrescine or spermidine concentration at which the initial uptake rate is half of V_{max}. The data are also represented using the Eadie-Hofstee plot (in which V_0 is plotted as a function of V_0/[polyamine]).

3. Results

3.1 Putrescine and spermidine uptake into isolated P. falciparum *parasites*

The ability of the intra-erythrocytic *P. falciparum* trophozoite to take up putrescine or spermidine across its plasma membrane was investigated in parasites functionally isolated from their host erythrocytes by saponin-permeabilization of the erythrocyte and parasitophorous vacuolar membranes. As illustrated in Fig. 1, both putrescine and spermidine were taken up by isolated, trophozoite-stage *P. falciparum* parasites. In paired experiments (i.e. putrescine and spermidine uptake performed with the same cell suspension, under conditions in which the extracellular polyamine concentration was 24 nM and 30 nM, for putrescine and spermidine, respectively), spermidine uptake occurred both at a higher initial rate than putrescine uptake (1.0 ± 0.4 pmol spermidine/(10^{10}cells . min) vs. 0.41 ± 0.13 pmol putrescine/(10^{10}cells . min), n = 7, P = 0.06, paired t-test), and to a significantly higher final distribution ratio than putrescine uptake. (2.4 ± 0.5 for spermidine vs.1.33 ± 0.22 for putrescine, n = 7, P = 0.04, paired t-test).

Polyamine uptake has been shown to be temperature-dependent in a variety of cells (Basselin et al., 2000; Fukumoto and Byus, 1996; Romero-Calderon and Krantz, 2006; Soulet et al., 2002). The same was found here to be true here in isolated *P. falciparum* parasites. Reduction of the temperature from 37°C to 22°C led to a significant decrease in the initial rate of uptake of both putrescine and spermidine, with the putrescine uptake rate at 22°C decreasing to 59 ± 6% of that at 37°C (n = 4, P = 0.03, unpaired t-test) and the spermidine uptake rate at 22°C decreasing to 42 ± 8% of that at 37°C (n = 6, P = 0.003, paired t-test, results not shown).

3.2 Kinetics of putrescine or spermidine uptake into isolated parasites

The uptake of [^{3}H]putrescine and [^{3}H]spermidine into isolated *P. falciparum* parasites was measured in the presence of a range of concentrations of unlabeled putrescine and spermidine,

respectively. Uptake was measured over a 15 min period which, for both polyamines, fell within the initial approximately linear phase of the uptake time-course (Fig 1).

Putrescine uptake appeared to obey Michaelis-Menten kinetics over the concentration range (0-15 mM; Fig. 2A). Fitting the Michaelis-Menten equation to the data range yielded an apparent K_m of 9.1 ± 1.2 mM and a V_{max} of 9.7 ± 2.2 µmol putrescine/(10^{10} cells . h) (n = 5; Fig. 2A). By contrast, spermidine uptake showed a non-linear dependence on concentration over a low concentration range (0-500 µM), but an approximately linear dependence on concentration over a higher concentration range (0-15 mM; Fig. 2B). Fitting the Michaelis-Menten equation to the low concentration range data yielded an apparent K_m of 0.42 ± 0.12 mM and a V_{max} of 0.14 ± 0.02 µmol spermidine/(10^{10} cells . h) (n = 5; Fig. 2B).

For both putrescine and spermidine, plotting those data that were fitted by the Michaelis-Menten equation as an Eadie-Hofstee plot revealed that for both polyamines the saturable component is itself composed of multiple kinetic components (insets to Figs. 2A and 2B, respectively).

3.3 Competition with putrescine and spermidine uptake into isolated parasites

The specificity of the mechanism(s) by which the parasites take up polyamines was investigated by assessing the ability of a range of polyamines and amino acids, each at an external concentration of 5 mM, to inhibit the uptake of [^{3}H]putrescine and [^{3}H]spermidine into isolated *P. falciparum* parasites. The results are represented in Fig. 3. Unlabelled putrescine, spermidine and a third polyamine, spermine, all inhibited the uptake of both [^{3}H]putrescine and [^{3}H]spermidine (n ≥ 3, all $P < 0.05$, Wilcoxon matched pairs test), with the tri- and tetravalent compounds spermidine and spermine causing a greater inhibition than the divalent compound putrescine. Ornithine, the amino acid precursor of putrescine, inhibited the uptake of both putrescine and (to a lesser extent) spermidine (n ≥ 3, all $P < 0.05$). Other basic amino acids - histidine, lysine and arginine - caused significant inhibition of the uptake of [^{3}H]putrescine (n = 5, all $P < 0.05$, Wilcoxon matched pairs test), but not that of spermidine (n = 6, $P \geq 0.05$, Wilcoxon matched pairs test). The neutral amino

acids leucine, tryptophan and glutamine, and the acidic amino acid glutamate, caused a <20% reduction in the uptake of the radiolabelled polyamines; in some cases (e.g. the apparent effect of tryptophan on the uptake of both putrescine and spermidine) the reduction reached statistical significance ($P < 0.05$, Wilcoxon matched pairs test) whereas in others (e.g. the small apparent inhibition of spermidine uptake by glutamate) it did not.

3.4 Effect of inhibition of polyamine biosynthesis on the uptake of putrescine and spermidine into isolated parasites

Polyamines are present at an estimated total concentration of 10 mM inside *P. falciparum* trophozoites (Teng et al., 2009). This can be reduced by polyamine biosynthesis inhibitors; incubation of parasites with the polyamine biosynthesis inhibitor DFMO leads to a ~20-fold decrease in putrescine levels and a ~10-fold decrease in spermidine levels in *P. falciparum*-infected erythrocytes (Assaraf et al., 1987b).

The effect of a DFMO-induced reduction of intracellular polyamine concentrations on the uptake of putrescine and spermidine was investigated, with the results shown in Fig. 4. Treatment of cells with DFMO (for 24 h, from early ring stage) resulted in a significant increase in the total uptake of both [^{3}H]putrescine or [^{3}H]spermidine into isolated parasites as measured over 60 min. In the case of [^{3}H]putrescine, the increase was approximately 2-fold. In the case of [^{3}H]spermidine the increase was approximately 4-fold. While there was no statistical difference between untreated and DFMO-treated cells in the initial rate of [^{3}H]putrescine uptake (1.12 ± 0.27 vs. 1.38 ± 0.29 pmol [^{3}H]putrescine/(10^{10} cells . min) for untreated and DFMO-treated cells, respectively; n = 7, $P = 0.6$, unpaired t-test), the initial rate of [^{3}H]spermidine uptake was significantly higher in the DFMO-treated cells than in the untreated cells (0.98 ± 0.09 pmol vs. 3.4 ± 0.8 pmol [^{3}H]spermidine/(10^{10} cells . min) for untreated and DFMO-treated cells, respectively; n = 5, $P = 0.01$, unpaired t-test, Fig. 4).

3.5 Na^+- and pH-dependence of putrescine or spermidine uptake

A number of experiments were carried out to investigate the effect of the extracellular ionic environment on the uptake of polyamines by the isolated parasite. Replacement of Na^+ with the inert organic cation NMDG in the extracellular medium (solution D), had no significant effect on the initial rate of uptake of either putrescine (n = 4, P = 0.9, unpaired t-test, results not shown) or spermidine (n = 6, P = 0.3, unpaired t-test, results not shown).

By contrast, as illustrated in Fig. 5, the uptake of both polyamines showed a marked dependence on the extracellular pH. For [^{3}H]putrescine, the accumulation measured at 30 min was significantly higher at pH 8.1 than at pH 7.1, and higher at pH 7.1 than at pH 6.1 (P = 0.009 and P = 0.12 respectively, n = 6, paired t-test). The initial rate of [^{3}H]putrescine uptake was significantly higher at pH 8.1 than at pH 7.1 (n = 6, P = 0.0495, paired t-test), and slightly (but not significantly) higher at pH 7.1 than at pH 6.1 (n = 6, P = 0. 0.32, paired t-test).

For [^{3}H]spermidine, the accumulation measured at 60 min, as well as the initial uptake rate, also decreased with decreasing pH from pH 8.1 to 6.1. The initial rate of [^{3}H] spermidine uptake was significantly higher at pH 8.1 than at pH 7.1 (n = 6, P = 0.02, paired t-test) and slightly (but not significantly) higher at pH 7.1 than at pH 6.1 (n = 6, P = 0.53, paired t-test). The accumulation measured at 60 min was significantly higher at pH 8.1 than at pH 7.1, and significantly higher at pH 7.1 than at pH 6.1 (P = 0.048 and P = 0.039, respectively, n = 6, paired t-test).

The increase in uptake of the two polyamines as the extracellular pH was increased might be due, at least in part, to changes in the protonation state of the two species. As the pH increases, so too does the proportion of each of the polyamines present in the unprotonated, and hence uncharged form. To investigate the possibility that the polyamines enter the cells as the uncharged species, perhaps via a process of simple diffusion through the lipid bilayer, we tested the effect of putrescine (10 mM), on the resting cytosolic pH (pH_{cyt}) of isolated parasites preloaded with the pH-

sensitive fluorescent indicator dye BCECF, comparing it with the effect of the weak base NH_4^+. The experiments was carried out at a pH_o of both 7.1 and 8.1 (i.e. the pH_o at which increased polyamine uptake was observed). The initial resting pH_{cyt} of the cells suspended at an external pH (pH_o) of 7.1 was 7.28 ± 0.03 (n = 4) and at pH_o = 8.1 was 7.690 ± 0.002 (n = 4). Addition of 10 mM NH_4Cl resulted in an immediate transient alkalinization of 0.072 ± 0.004 pH units at pH_o 7.1 (n = 4, *P* = 0.0004, paired t-test), and an alkalinization of 0.24 ± 0.01 pH units at pH_o 8.1 (n = 4, *P* = 0.0002, paired t-test). The NH_4-induced alkalinization was a result of the diffusion of the neutral uncharged (NH_3) species into the parasite and its 'capture' of cytosolic H^+ ions in the conversion of NH_3 to NH_4^+. By contrast, there was no significant alkalinization observed following the addition of putrescine at either pH_o 7.1 (n = 4, *P* = 0.06, paired t-test) or pH_o8.1 (n = 4, *P* = 0.14, paired t-test, results not shown). There was therefore no evidence for the polyamines entering the parasites as the neutral uncharged species at either pH.

3.6 Membrane potential dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake

In isolated parasites, an increase in the extracellular pH induces a plasma membrane hyperpolarization, whereas a decrease in the extracellular pH induces a depolarization (Allen and Kirk, 2004). The possibility therefore arises that the observed pH-dependence of polyamine uptake (Fig. 5) might be due, at least in part, to a dependence of polyamine uptake on membrane potential. The membrane potential of asexual blood-stage parasites originates primarily from the extrusion of H^+ via an electrogenic V-type H^+-ATPase on the parasite surface and is moderated by the influx of K^+ via conductive K^+ channels (Allen and Kirk, 2004). To assess the membrane potential dependence of polyamine import, the uptake of both putrescine and spermidine was measured under conditions in which the membrane potential was manipulated by varying the extracellular K^+ concentration, both in the presence and absence of the K^+ ionophore valinomycin, and by the addition of the V-type H^+-ATPase inhibitor concanamycin A. Removal of extracellular K^+ results in a membrane hyperpolarization, and this is accentuated in the presence of valinomycin (Allen and Kirk, 2004). As is shown in Fig. 6, removal of extracellular K^+ resulted in increased uptake of the two polyamines (measured over 15 min) with a further increase seen on addition of

valinomycin. Under both conditions, the increase seen for spermidine (with three positive charges) was greater than that seen for putrescine (with two positive charges). Although the increases in putrescine uptake seen in cells suspended in K^+-free medium (both in the absence and presence of valinomycin) were reproducible they did not reach statistical significance (n = 5, $P = 0.07$ in each case, Wilcoxon matched pairs test). The increase in spermidine uptake seen in cells suspended in K^+-free medium in the presence of valinomycin was statistically significant (n = 6, $P = 0.04$, Wilcoxon matched pairs test) whereas that seen in the absence of valinomycin was not (n = 6, $P = 0.7$, Wilcoxon matched pairs test).

Conversely, increasing the extracellular K^+ from the normal plasma value of ~5 mM results in a membrane depolarization, and this is again accentuated in the presence of valinomycin (Allen and Kirk, 2004). Increasing the extracellular K^+ to 130 mM decreased the uptake of the two polyamines (measured over 15 min) significantly (n ≥ 3, both $P < 0.05$, Wilcoxon matched pairs test), with a further decrease seen on addition of valinomycin (Fig. 6). Again, the effect was more pronounced for spermidine than for putrescine. Similarly, depolarization of the plasma membrane by the addition of concanamycin A caused a significant decrease (n ≥ 3, both $P < 0.05$) in the uptake of both polyamines, and the combination of concanamycin A and an increased extracellular K^+ concentration together caused an even larger decrease (n ≥ 3, both $P < 0.05$), with the decrease for spermidine again greater than that for putrescine.

4. Discussion

The major finding of this study is that the polyamines putrescine and spermidine are taken up by the intraerythrocytic malaria parasite via a mechanism that is influenced by the extracellular pH and by parasite's membrane potential. The triamine spermidine was taken up by isolated parasites at a faster rate, and accumulated to a higher level, than the diamine putrescine. A higher rate of uptake of spermidine, than putrescine, has been reported previously for rat liver mitochondria (Toninello et al., 1992). As has been reported for the uptake of polyamines into other parasites (e.g. *Leishmania* spp; (Basselin et al., 2000)) the uptake of putrescine and spermidine into the *P. falciparum* trophozoite was temperature-dependent, with the rate decreasing by approximately two-fold on reduction of the temperature from 37°C to 22°C.

Polyamines interact electrostatically with negatively charged macromolecules within the cell, including phospholipids, proteins and nucleic acids (Wallace et al., 2003). They are also metabolized, with some interconversion between the different polyamines and some intracellular spermidine undergoing conversion to the amino acid hypusine, present in eukaryotic initiation factor-5A (eIF-5A) (Molitor et al., 2004). The measured uptake of polyamines reflects the combined effects of transport into the parasite, accumulation due to binding to intracellular sites, and intracellular metabolism, and it is important to recognize that the characteristics of the measured uptake may reflect each of these processes (Kirk et al., 2009). In particular, the multiple components (high and low affinity) revealed in the kinetic experiments in which polyamine uptake into *P. falciparum* parasites was measured over a wide concentration range are likely to reflect the affinities of both transport and binding to intracellular sites, and it is not straightforward to distinguish between them. Previous studies of polyamine uptake in other cell types have reported the presence of multiple kinetic components. For example, in *Xenopus laevis* oocytes, putrescine uptake has at least two distinct kinetic components (Fukumoto and Byus, 1997). Similarly, putrescine uptake in erythrocytes has both a non-saturable component (perhaps indicative of diffusion through the membrane bilayer) as well as a saturable component (Fukumoto and Byus, 1996).

The competition experiments revealed cross-competition between putrescine and spermidine, as well as competition by a third polyamine, spermine, with the uptake of both putrescine and spermidine into *P. falciparum* parasites. As with the kinetics of uptake, it must be recognized that the competition between the different polyamines may reflect, at least in part, competition for binding to intracellular sites. Nevertheless, the data are consistent with the different polyamines sharing one or more transport pathways. The finding that the basic amino acids ornithine, histidine, arginine and lysine all inhibited the uptake of putrescine is consistent with the dibasic polyamine and the basic amino acids sharing a common transport pathway. However, the finding that (with the exception of ornithine) the basic amino acids had little effect on spermidine uptake argues against the operation of a single transport pathway and is, again, consistent with there being multiple components to the measured uptake of polyamines. Some cell types, (e.g. murine leukaemia L1210 cells (Porter et al., 1984) and hamster amelanocytic melanoma AMEL-3 cells (García-Fernández et al., 2005) are thought to have a single, shared polyamine transporter, while for others (e.g. human leukaemia HL-60 cells (Palmer and Wallace, 2010), CHO cells (Xie et al., 1997), or *T. cruzi* parasites (Hasne et al., 2010)) there is evidence for separate transport systems for putrescine and spermidine and spermine.

The finding here that the *P. falciparum* parasite has the capacity to take up exogenous polyamines provides an explanation for the previous observation that the cytostatic effects of the polyamine synthesis inhibitor DFMO can be overcome by the addition of polyamines to the extracellular medium (Assaraf et al., 1987a). DFMO reduces the intracellular concentration of polyamines. The marked increase in the uptake of radiolabelled putrescine and spermidine in cells pretreated with DFMO most likely reflects the increased availability of intracellular polyamine binding sites in the polyamine-depleted cells. A similar increase in the uptake of exogenous polyamines following polyamine depletion has been observed in AMEL-3 (García-Fernández et al., 2005), Ehrlich ascites carcinoma cells (Alhonen-Hongisto et al., 1980) and HL-60 cells (Walters and Wojcik, 1994). The finding that the initial rate of uptake of [^{3}H]spermidine uptake was significantly higher in the DFMO-treated cells than in untreated cells raises the possibility that the parasite might respond

to polyamine depletion by the up-regulation of one or more transport pathways. However, a previous analysis of the transcriptional profile of intra-erythrocytic *P. falciparum* following DFMO-induced polyamine depletion did not identify altered expression of genes encoding proteins with similarity to known polyamine transporters (van Brummelen et al., 2009).

Polyamine uptake into isolated parasites was not dependent on extracellular Na^+ but showed a pronounced dependence on the extracellular pH. In particular, polyamine uptake increased markedly when the extracellular pH was increased to 8.1, raising the possibility that the compounds enter the cell in the neutral unprotonated form (the relative proportion of which increases with increasing pH). The observation that the uptake of a high (10 mM) concentration of putrescine to an isolated parasite suspension had no significant effect on cytosolic pH (contrasting with the substantial alkalinization seen on addition of an equivalent concentration of NH_4^+) argues against there being a rapid diffusion of unprotonated putrescine across the membrane bilayer, both at pH 7.1 and pH 8.1. However, it should be noted that the parasite does have pH regulatory systems that allow it to counter a cytosolic alkalinization (Henry et al., 2010). The possibility therefore cannot be excluded that a fraction of the polyamines do enter via diffusion, much more slowly than NH_3/NH_4^+, and that the parasite effectively counters (and therefore masks) the potential pH changes.

Increasing the extracellular pH results in a membrane hyperpolarization whereas decreasing the extracellular pH results in depolarization (Allen and Kirk, 2004). In experiments in which the parasite's membrane potential was varied by manipulating the extracellular ion concentration and membrane permeability, a membrane hyperpolarization was shown to result in an increase in polyamine uptake, while membrane depolarization resulted in a decrease. This is consistent with the uptake process involving a net influx of positive charge, as would be the case if the polyamines enter the parasite in the charged (i.e. protonated) form. The observation that the effects of membrane potential on the tribasic compound spermidine (which bears three positive charges in the protonated form) were more pronounced than those on the dibasic compound putrescine (which bears two positive charges in the protonated form) is again consistent with this view. The

observed effect of pH on polyamine uptake may therefore be due, at least in part, to the pH dependence of the parasite's membrane potential.

In summary, both putrescine and spermidine are taken up by intra-erythrocytic *P. falciparum* parasites from the external environment. The possibility cannot be excluded that a component of this is via the diffusion of the non-protonated forms of the molecules via the membrane bilayer, with any such component increasing with increasing extracellular pH (as the relative concentration of the uncharged species increases). Nevertheless the membrane potential dependence of polyamine uptake and the fact that this dependence was more pronounced for the tribasic polyamine spermidine than for the dibasic polyamine putrescine, are consistent with the hypothesis that the compounds enter the parasite as positively charged species, with uptake 'energised' by the parasite's large inwardly negative membrane potential (estimated to be approximately -95 mV under physiological conditions (Allen and Kirk, 2004)). The finding of cross-competition in the uptake of the different polyamines is consistent with there being one or more shared transporters. The observation of saturable components in the uptake of both putrescine and spermidine is also consistent with the involvement of one or more saturable transporters; however, the complex nature of the uptake process involving transport into the cell and sequestration at intracellular binding sites does make the interpretation of such data difficult. The molecular identity of any such polyamine transporter in the parasite remains elusive. Several known polyamine transporters, such as the bacterial Pot E (Kashiwagi et al., 2000), *S. cerevisiae* Agp2p (Aouida et al., 2005), UGA4 (Uemura et al., 2004) and *L. major* LmPot1 (Haider et al., 2005) are members of the amino acid/polyamine/organocation (APC) superfamily of transporters. While preliminary investigations show that the *P. falciparum* genome encodes at least one APC superfamily transporter protein, the substrate specificity of this protein has yet to be established.

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Figure legends

Figure 1: Time courses for the uptake of [^{3}H]putrescine (■) and [^{3}H]spermidine (□) by isolated *P. falciparum* trophozoites at 37°C. The extracellular concentrations of the two polyamines were 24 nM and 30 nM, respectively. Polyamine uptake is expressed in terms of distribution ratio (i.e. the apparent intracellular concentration of radiolabelled polyamine relative to the extracellular concentration). The data are averaged from seven independent (paired) experiments and are shown ± S.E.

Figure 2: Kinetics of the uptake of (A) putrescine and (B) spermidine into isolated *P. falciparum* trophozoites. The cells were suspended in solution A and uptake was measured over 15 min at 37°C. The putrescine data were fitted to the Michaelis-Menten equation: polyamine influx = V_{max} [polyamine]/(K_m + [polyamine]), with K_m = 9.1 ± 1.2 mM and V_{max} = 9.7 ± 2.2 μmol putrescine/(10^{10} cells . h). In the case of spermidine the data obtained over the concentration range 1-500 μM were fitted to the Michaelis-Menten equation (K_m = 0.42 ± 0.12 mM and V_{max} = V_{max}=0.14 ± 0.02 μmol spermidine/(10^{10} cells . h) whereas those obtained over the higher concentration range (0.5 - 15 mM) were fitted to a straight line (influx = a [polyamine], where a = 0.14 μmol spermidine/(10^{10} cells . h . mM)). In the insets the putrescine data obtained over the concentration range 0-15 mM (A), and the spermidine data obtained over the concentration range 1-500 μM (B), are represented using Eadie-Hofstee plots in which V_0 (μmol polyamine/(10^{10} cells . h)) is plotted as a function of V_0/[polyamine] (with [putrescine] in mM and [spermidine] in μM). The non-linearity of the Eadie-Hofstee plots indicates that for both polyamines the saturable component is itself composed of multiple kinetic components. The data were averaged from 5 independent experiments and are shown ± SE.

Figure 3: Effect of a range of (unlabelled) polyamines and amino acids, each at a concentration of 5 mM, on the uptake of [^{3}H]putrescine and [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites. Uptake was measured over 30 min at 37°C. The

concentrations of [^{3}H]putrescine and [^{3}H]spermidine were 24 and 30 nM, respectively. Uptake is expressed as percentage of that measured in the absence of competing substrate. Statistical significance was determined with a Wilcoxon Matched Pairs test, with an asterisk denoting $P<0.05$. [^{3}H]Putrescine uptake is indicated by black bars, with the data averaged from 5 independent experiments and shown ± SE. [^{3}H]Spermidine uptake is indicated by grey bars, with the data averaged from 6 independent experiments and shown ± SE.

Figure 4: Effect of DFMO-pretreatment on the uptake of polyamines by isolated *P. falciparum* trophozoites. Parasitised erythrocytes, initially in the ring-stage (~6 h post-invasion), were incubated with 2 mM DFMO for 24 h (allowing the parasites to mature to the trophozoite stage). Treatment with DFMO has been shown previously to decrease the levels of polyamines within the parasite (Assaraf et al., 1987a). Uptake of (A) [^{3}H]putrescine and (B) [^{3}H]spermidine (present at concentrations of 24 and 30 nM, respectively) was measured in isolated DFMO-treated (▲,△) and untreated (■,□) parasites at 37°C. The uptake of each polyamine is expressed in terms of pmol/10^{10} cells rather than distribution ratio as calculation of the latter requires a knowledge of cell volume and the effect of DFMO pretreatment on parasite volume is unknown.

Figure 5: Effect of pH on the uptake of polyamines by isolated *P. falciparum* trophozoites. Isolated parasites were suspended in solution E in which the pH was adjusted to 6.1 (◆,◇),7.1 (■,□) or 8.1 (●,○) and the uptake of (A) [^{3}H]putrescine (present at 5 nM) and (B) [^{3}H]spermidine (present at 30 nM) was measured at 37°C. The [^{3}H]putrescine and [^{3}H]spermidine uptake data were averaged from six independent experiments and are shown ± SE.

Figure 6: Effect of membrane potential perturbations on the uptake of polyamines by isolated *P. falciparum* trophozoites. Uptake was measured over 15 min at 37°C. The cells were hyperpolarized by being suspended in Solution B (containing 0 K^+) for 30 min prior to adding the [^{3}H]putrescine (24 nM) or [^{3}H]spermidine (30 nM). A further hyperpolarization was achieved by the addition of the K^+ ionophore valinomycin (1 μM, added to cells pre-incubated for 30 min in Solution

B) simultaneously with the [^{3}H]putrescine or [^{3}H]spermidine. The cells were depolarized by being suspended in Solution C (containing 150 mM K^+) for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine. A further depolarization was achieved by the addition of the K^+ ionophore valinomycin (1 μM, added to cells pre-incubated for 30 min in Solution C) simultaneously with the [^{3}H]putrescine or [^{3}H]spermidine. Cells were also depolarized using the V-type ATPase inhibitor concanamycin A (100 nM). Maximum depolarization was achieved by adding concanamycin A to cells suspended in a high-K^+ medium (solution C). Uptake is expressed as a percentage of that measured under control conditions and is shown ± SE. Statistical significance was determined using a Wilcoxon Matched Pairs test, with an asterisk denoting $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars, with the data averaged from 5 independent experiments and shown ± SE. [^{3}H]Spermidine uptake is indicated by grey bars, with the data averaged from six independent experiments and shown ± SE.

Figure 1

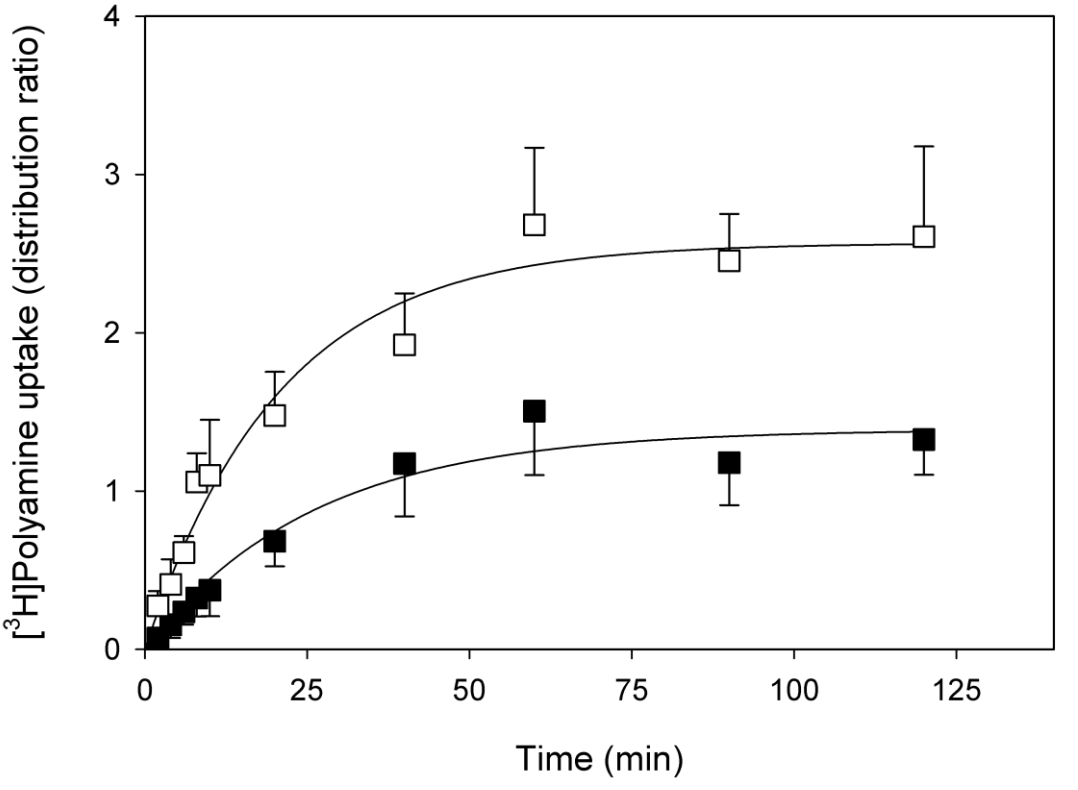

[3H]Polyamine uptake (distribution ratio)
0
1
2
3
4
0
25
50
75
100
125
Time (min)

Figure 2

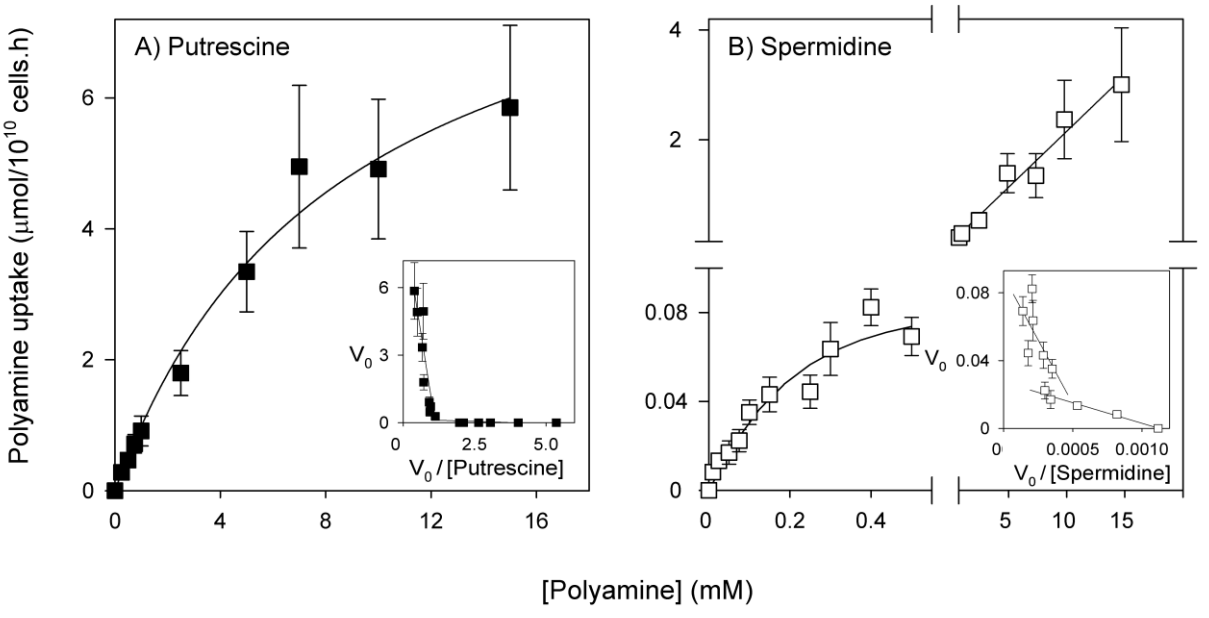

A) Putrescine
B) Spermidine
Polyamine uptake (μmol/10^10 cells.h)
[Polyamine] (mM)
V0 / [Putrescine]
V0 / [Spermidine]
V0

Figure 3

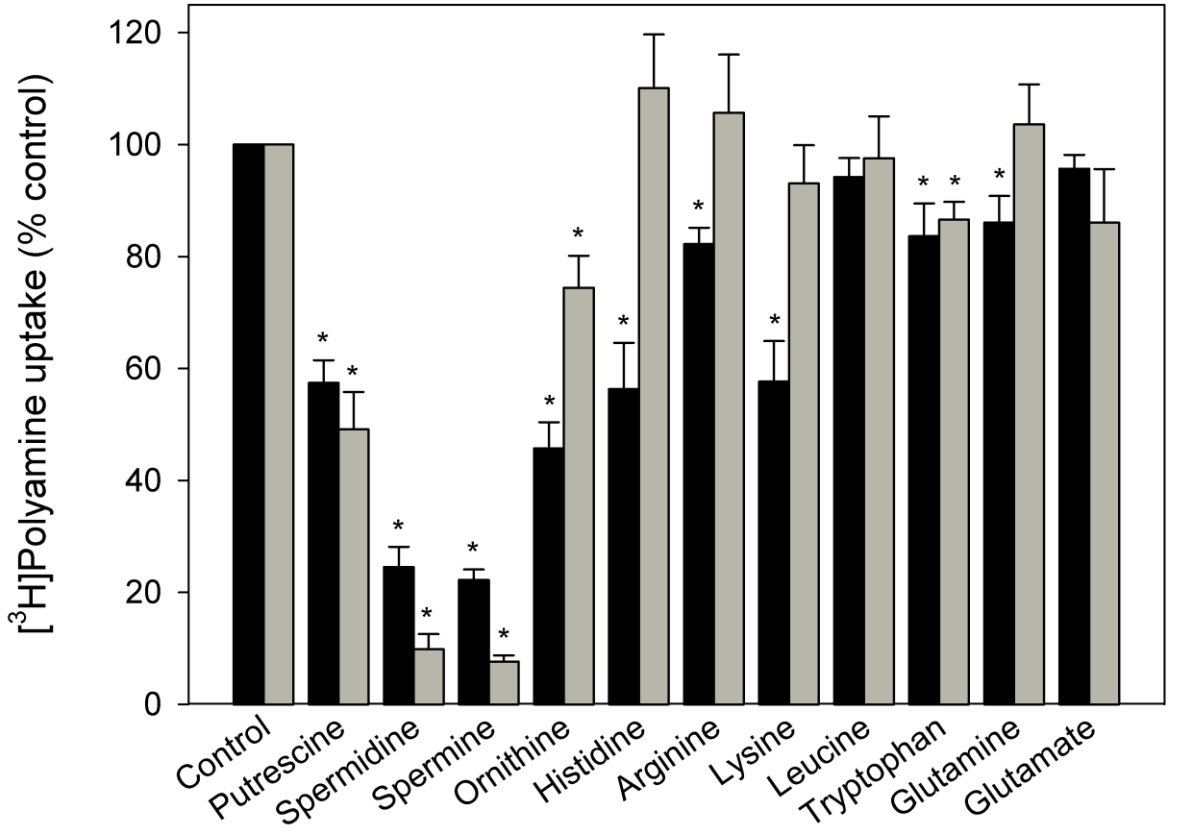

[3H]Polyamine uptake (% control)
120
100
80
60
40
20
0
Control
Putrescine
Spermidine
Spermine
Ornithine
Histidine
Arginine
Lysine
Leucine
Tryptophan
Glutamine
Glutamate

Figure 4

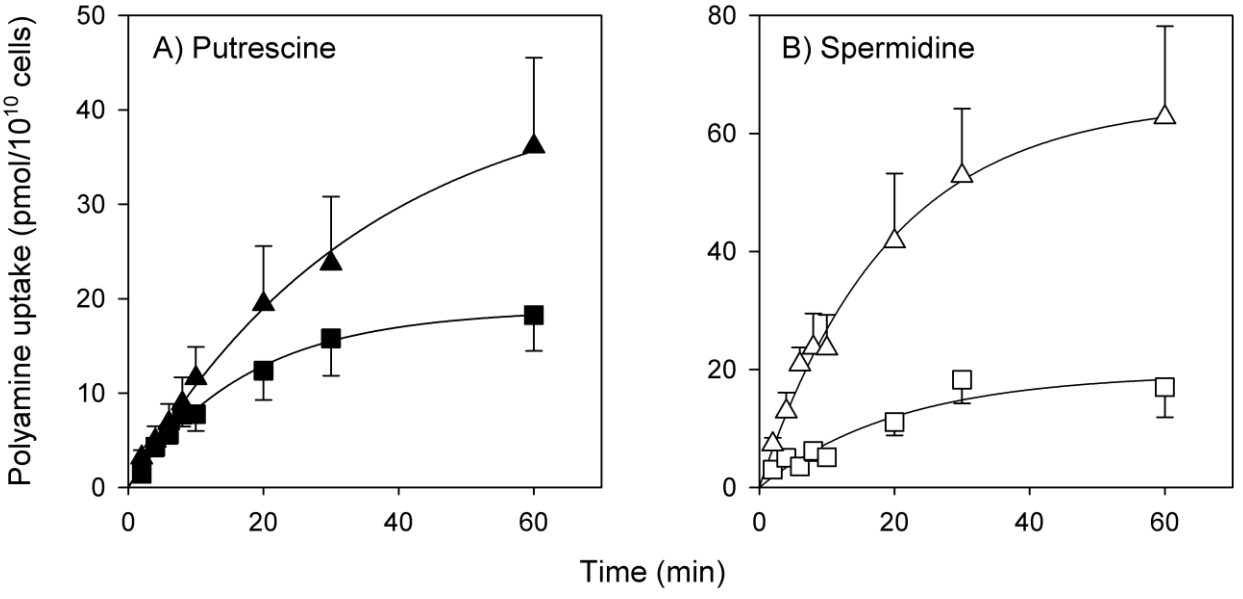

A) Putrescine
B) Spermidine
Polyamine uptake (pmol/10^10 cells)
Time (min)
0
10
20
30
40
50
60
80
0
20
40
60

Figure 5

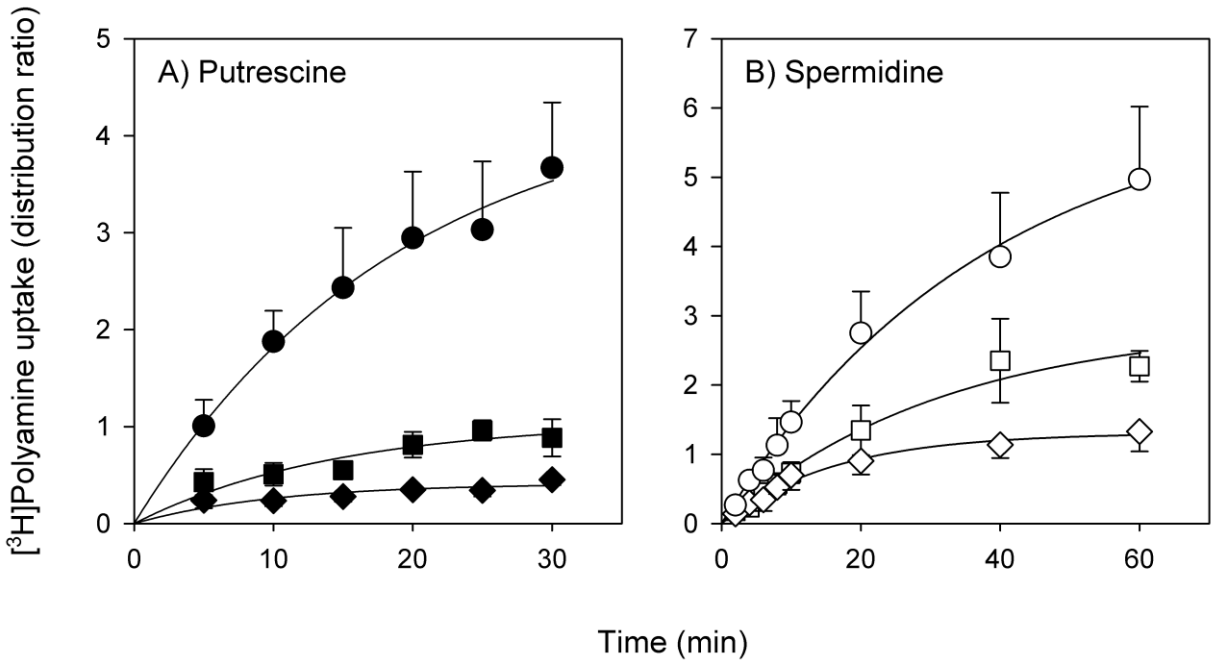
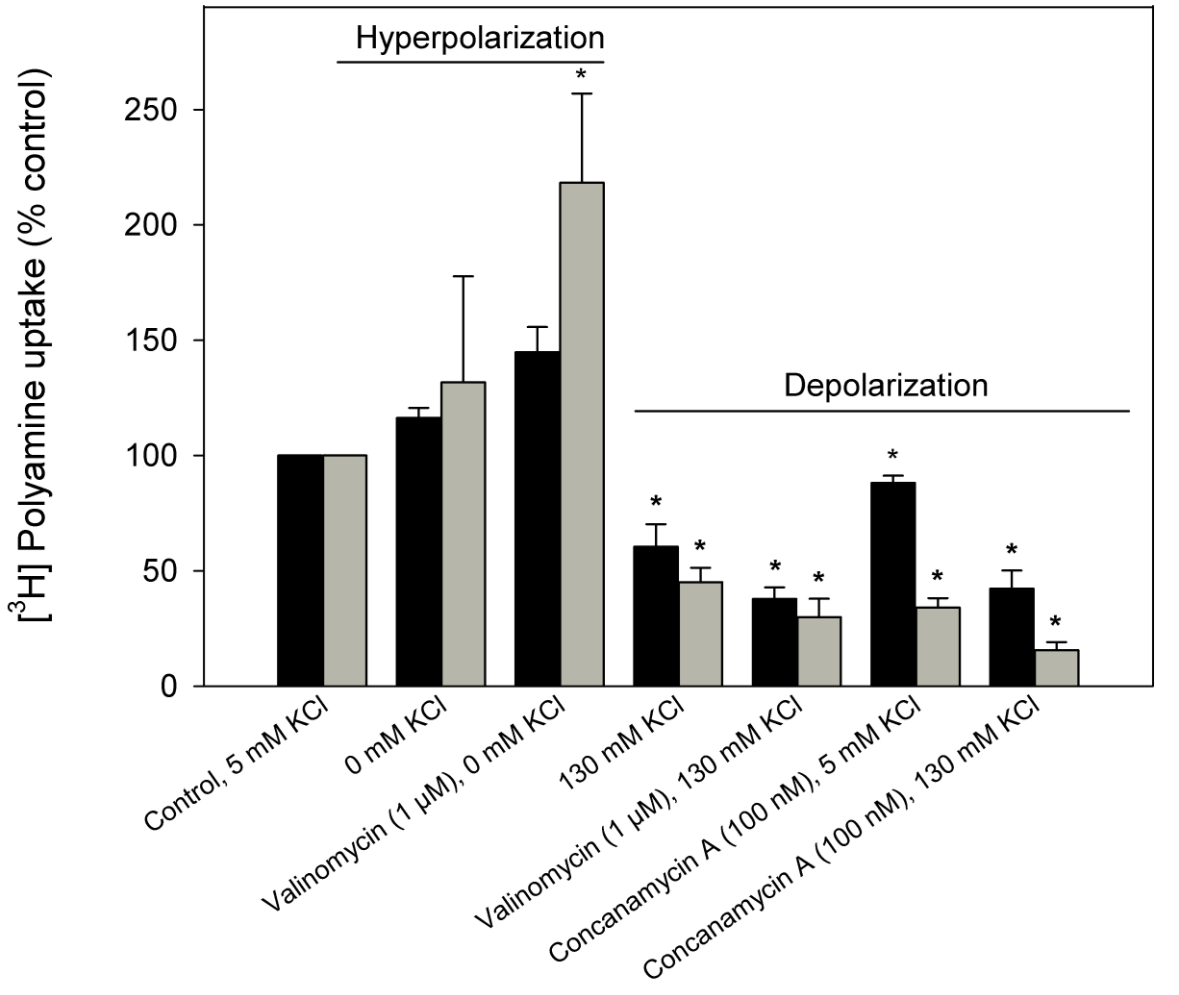

A) Putrescine
B) Spermidine
[3H]Polyamine uptake (distribution ratio)
Time (min)
0
1
2
3
4
5
6
7
0
10
20
30
40
60

Figure 6


Hyperpolarization
Depolarization
[3H] Polyamine uptake (% control)
0
50
100
150
200
250
*
Control, 5 mM KCl
0 mM KCl
Valinomycin (1 µM), 0 mM KCl
130 mM KCl
Valinomycin (1 µM), 130 mM KCl
Concanamycin A (100 nM), 5 mM KCl
Concanamycin A (100 nM), 130 mM KCl