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Selective Sites for Polyamine Binding to Rabbit Intestinal Brush-Border Membranes

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ABSTRACT. The intestinal polyamine transporters have not yet been identified. Our aim was to characterize specific polyamine binding sites in rabbit intestinal brush-border membranes (IBBM) as a starting step for identification of polyamine transporters. This was investigated at 4° and at low membrane concentration. Saturation isotherms for [^{3}H]putrescine (PUT) binding indicated a single population of sites (puT) with a dissociation equilibrium constant K_d of 3.8 μM and a density of sites B_{max} of 58 pmol/mg of protein. [^{3}H]spermidine (SPD) binding also involved only one class of sites (spD), albeit with a lower affinity (K_d = 106 μM) and higher abundance (B_{max} = 1240 pmol/mg of protein) than puT. On the contrary, [^{14}C]spermine (SPM) bound two classes of sites (spM_1 and spM_2) differing in their affinity (K_d = 2.5 and 31.4 μM) and abundance (B_{max} = 467 and 1617 pmol/mg of protein, respectively). Membrane association of SPM at 4° was much faster than that of SPD and PUT, both of which proceeded at a similar rate. In contrast to PUT and SPD dissociation, SPM dissociation at 23° did not follow a first-order reaction. Specifically bound [^{3}H]PUT, unlike [^{3}H]SPD and [^{14}C]SPM, dissociated at 23° independently of the addition of nonradioactive polyamine. Methylglyoxal-*bis*-(guanylhydrazone) was an extremely potent inhibitor of PUT binding (K_i = 3.2 ± 1.5 nM), but as with PUT and cadaverine (CAD), it did not alter [^{3}H]SPD and [^{14}C]SPM binding substantially. The intestinal brush-border membrane may contain at least three sites specific for polyamine binding and exhibiting different ligand selectivity. Site puT might be associated with the transport system already described for intestinal uptake of PUT. BIOCHEM PHARMACOL **56**;4:517–526, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. putrescine; spermidine; spermine; binding; polyamine analogs

The polyamines SPD§ and SPM and their precursor PUT are low molecular weight, ubiquitously distributed organic cations that play an essential role in cell growth [1]. Mammalian cells possess two different pathways to regulate their polyamine content, one by enzymatic synthesis and interconversion and the other by membrane transport [2, 3]. The polyamine metabolic pathways have been intensively studied, and the involved regulatory enzymes, ornithine decarboxylase (ODC), S-AMDC, and spermidine/spermine N^1-acetyltransferase (SSAT) have been characterized at both the protein and gene levels [4]. On the other hand, there are as yet almost no biochemical and molecular data available on polyamine transport systems in mammals [5, 6], in contrast with *Escherichia coli* where several classes of polyamine transporters have been cloned [7]. The physiological significance of polyamine transport systems in higher organisms is obvious from the finding that cellular uptake is increased after inhibition of endogenous polyamine biosynthesis [8] or stimulation of cell growth [9–11]. Additionally, the pathways for both polyamine transport and metabolism are regulated by antizyme, a polyamine-inducible protein that exhibits a dual function by directing the rate of ornithine decarboxylase degradation and negatively regulating polyamine transport [12, 13].

In addition to intracellular biosynthesis, food [14] and gut bacterial microflora [15] provide mammalian tissues with substantial amounts of polyamines. The gastrointestinal tract has been observed to play an essential role in polyamine supply to normal or neoplastic body tissues [16]. Polyamines were found to readily cross the intestinal epithelium in segments of rabbit gut mounted in Ussing chambers [17]. Kinetic studies with IEC-6 cultured rat intestinal cells [18] and rabbit IBBM vesicles [19, 20] provided evidence for the existence of polyamine transport systems in the apical membrane of enterocytes. These studies and others [21, 22] also brought information about the inorganic cation dependence of intestinal uptake of polyamines, suggesting that it might be driven by the membrane potential. In particular, externally bound divalent cations, unlike Na^+ and H^+, appeared to be essential for PUT uptake in IBBM vesicles [19], as well as in human breast cancer cells [23] and fibroblasts [24]. Studies with

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§ *Abbreviations*: CAD, cadaverine; IBBM, intestinal brush-border membrane; MGBG, methylglyoxal-*bis*-(guanylhydrazone); PUT, putrescine; S-AMDC, S-adenosylmethionine decarboxylase; SPD, spermidine; and SPM, spermine.

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IEC-6 cells have additionally shown that PUT transport might also be dependent on the intracellular Ca^{2+} pool through a calmodulin-dependent mechanism [25].

Information on the nature of the polyamine transport system(s) in the apical membrane of intestinal cells is still scarce. We recently observed that [^{3}H]PUT influx in IBBM vesicles could not be transstimulated by intravesicular unlabeled PUT [19]. PUT transport may therefore involve binding to a single class transport system that behaves as a channel-like uni-, sym-or anti-porter rather than a polyamine translocating membrane carrier. In addition, inhibition studies with structural analogs of polyamines suggested that binding of PUT to its transport system may include a relatively large hydrophobic cavity in the vicinity of the anionic residues interacting with the amino groups of the ligand [19]. Although much less efficient than CAD, SPD and SPM were also competitors of PUT for uptake by IBBM vesicles. However, it appears from previous studies with undifferentiated intestinal cells [18,25] and isolated enterocytes [26, 27] that both SPD and SPM share a transport system different from the PUT-preferring transport system, in agreement with findings in many other mammalian cells [3, 28–32]. However, whether the enterocyte apical membrane contains transport systems other than the PUT-preferring transport system has as yet not been directly determined. In the present study, we characterized several sites specific for binding of polyamines in the IBBM of the rabbit. Saturation isotherms, association and dissociation kinetics, and competition between polyamines were monitored at 4° in order to minimize both catabolism and transport of these polycations, which are known to be temperature-dependent processes [15, 19–21, 26, 27]. The association of the different binding sites identified here with the currently known mechanisms of intestinal absorption will be discussed.

MATERIALS AND METHODS

Materials

[1,4(n)-^{3}H]PUT dihydrochloride (38.7 Ci/mmol) and [1,4(n)-^{3}H]SPD dihydrochloride (20.4 Ci/mmol) were purchased from DuPont/NEN. [1,4-^{14}C]SPM tetrahydrochloride (110 mCi/mmol) was provided by Amersham. MGBG and all unlabeled polyamines were obtained from Sigma. All other reagents and chemicals were of analytical grade and obtained from Sigma or Fisher. Nitrocellulose filters (pore size = 0.65 μm, diameter = 25 mm) were purchased from Millipore.

Membrane Isolation

Male New Zealand White rabbits were anesthetized with ketamine, then killed by intracardiac injection of saturated KCl solution. IBBM were isolated from intestinal mucosa by differential centrifugation in the presence of $CaCl_2$, as described in [19]. The final pellet of IBBM was suspended in a 10 mM Hepes-Tris buffer (pH 7.4) containing 300 mM D-mannitol. This suspension was aliquoted, stored in liquid nitrogen and used over a period of 2 months. Membrane protein concentrations were measured according to Bradford [33], with ovalbumin as a standard. The purity of freshly prepared IBBM was assessed from intestinal brush-border marker enzyme assays [19–22]. Specific activities of alkaline phosphatase and aminopeptidase *N* were increased by ~30- and 20-fold, respectively, in IBBM compared with the initial mucosa homogenate. Additionally, the preparation employed was essentially free of contamination by the basolateral membrane marker enzyme Na^+/K^+ ATPase, as shown by the finding of a ~ four-fold decrease in Na^+/K^+ ATPase specific activity in IBBM relative to the homogenate.

Radiometric Assay of Polyamine Binding

To measure labeled polyamine binding, IBBM (0.25–3.0 mg/mL) were incubated at 4° in a 10 mM Hepes-Tris buffer (pH 7.4) containing 50 mM NaCl, 200 mM D-mannitol and various concentrations of ligand ([^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM). In some experiments, a given concentration of unlabeled competitor was also present in the medium (total volume = 110 μL). To terminate the incubations, 1 mL of a pH 7.4-buffered solution (100 mM NaCl, 100 mM D-mannitol, 10 mM HEPES-Tris) was added to the medium and the mixture was rapidly filtered under vacuum through a nitrocellulose filter. The filters were washed twice with 4 mL of termination buffer, and their radioactivity was measured by liquid scintillation in 10 mL of Ecoscint A (National Diagnostics). Specific binding of labeled polyamine was obtained by subtracting nonspecific binding from total binding as determined in parallel tubes containing 10 mM unlabeled polyamine.

Data Analyses

Binding assays were performed in triplicate with membrane suspensions obtained from two different rabbits. Data are usually expressed as picomoles of bound ligand per mg of protein and presented as means ± SD, the latter being omitted however in figures when smaller than symbol size.

For the determination of saturation isotherms, the ligand concentration was varied by adding increasing concentrations of nonradioactive ligand to a fixed amount of [^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM. Saturation isotherms were analyzed by iterative curve fitting of specific binding data to equations modeling one or two saturable sites using the SigmaPlot software program (Jandel Scientific). Values for the equilibrium dissociation constant K_d and the density of binding sites B_{max} obtained by iterative curve fittings, were comparable to values obtained by Scatchard analysis.

Total binding of polyamines to the membranes remained less than 3% of their amount in the incubation medium (not illustrated). Thus, free ligand concentration could be considered to remain close to total ligand concentrations with time, and the initial time courses of association could

be analyzed as pseudo-first-order reactions described by Equation 1,

$$\log(100-100 \times [B_t]/[B_{eq}]) = -(k_{+1}[L] + k_{-1})t,$$

in which $[B_{eq}]$ and $[B_t]$ are the amounts of bound polyamine at equilibrium and at a given time, respectively: [L], the free ligand concentration; k_{+1}, the apparent association rate constant; and k_{-1}, the apparent dissociation rate constant.

Finally, competition studies were carried out in the presence of a fixed amount of radioactive ligand (0.95 μM [^{3}H]PUT, 49 μM [^{3}H]SPD or 23 μM [^{14}C]SPM) and increasing concentrations of the test competitor. The inhibition constant value K_i, of the latter was calculated using a versatile computer program, EBDA-LIGAND [34].

RESULTS

Polyamine Binding at Equilibrium

PUT, SPD and SPM are essentially transported into an osmotically active intravesicular space when incubated with IBBM vesicles at 37° [19–22]. While PUT undergoes rapid enzymatic oxidation under these conditions [19], we found here that it was not metabolized during 24-hr incubations at 4° (data not shown). Additionally, we observed that membrane binding of all three polyamines remained higher than 80% of total accumulation under the latter conditions, thus allowing for accurate measurements. To determine values of equilibrium dissociation constants (K_d) of polyamines, we first measured binding of 0.01–1 μM ligand as a function of membrane concentration in the 0.2–3.0 mg of protein/mL range for incubations of 18 hr at 4°. For instance, at 0.1 μM [^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM, the amount of bound ligand increased up to a value of 25, 6 or 38% of the total polyamine amount that was reached at a membrane concentration of 1.5, 1.0 or 0.75 mg of protein/mL, respectively. A membrane concentration of 0.5 mg of protein/mL was eventually selected for performing all binding assays as a compromise between the limits of detection of ^{3}H and ^{14}C radioactivity by liquid scintillation, and the requirement that concentration of ligand binding sites must be close to K_d.

The time courses of total binding of polyamines at 4° were exponential over a ligand concentration range of 0.1–700 μM (not illustrated). Times ranging between 7 min (at 700 μM SPM) and 25 hr (at 0.1 μM PUT) were needed for complete saturation of specific binding sites. Binding was stable for at least 30 hr at 4°, but an incubation duration of 18 hr was chosen to determine saturation isotherms, due to the possibility of deleterious effects of longer incubation times on membrane integrity. In addition, at concentrations higher than 25 μM, PUT binding became mainly nonspecific under these conditions, in contrast to SPD or SPM binding that remained mostly specific through all the range of concentrations used. Figures 1A, 1C and 1E depict saturation isotherms of specific (solid lines) and nonspecific (dotted lines) binding of PUT, SPD and SPM, respectively, for ligand concentrations in which the specific to nonspecific binding ratio was ≥1. Analysis of specific binding of PUT and SPD by iterative curve fitting or Scatchard plot (Fig. 1B and 1D) indicated a single class of binding sites puT and spD, respectively. Sites puT exhibited a higher affinity (K_d = 3.8 vs 106 μM) and a lower abundance (B_{max} = 58 vs 1240 pmol/mg of protein) than sites spD. Similar data analysis (Fig. 1F) showed that, in contrast to PUT and SPD, SPM could specifically bind two different sites on the membranes, one (spM_1) exhibiting a higher affinity (K_d = 2.5 vs 31.4 μM) but a lower abundance (B_{max} = 467 vs 1617 pmol/mg of protein) than the other (spM_2).

Kinetics of Association and Dissociation

The time course of association for radiolabeled PUT, SPD or SPM to membranes was studied with various concentrations of each ligand ranging from 1 to 25 μM, 24 to 477 μM or 1 to 243 μM, respectively. When log (100–100 × $[B_t]/[B_{eq}]$) was plotted as a function of time (see Eqn 1 in Materials and Methods), a straight line with a slope (k*) proportional to $k_{+1}[L] + k_{-1}$ was obtained with all concentrations of PUT or SPD (coefficients of correlation ≥ 0.94). This is illustrated in Fig. 2A and B for the highest and lowest polyamine concentrations used. Values for $k_{+1,\ put}$ and $k_{-1,\ put}$ or $k_{+1,\ spd}$ and $k_{-1,\ spd}$ that were determined from a replot of $k^{*,\ put}$ or $k^{*,\ spd}$ as a function of concentration of either [^{3}H]PUT (Fig. 2D) or [^{3}H]SPD (Fig. 2E), respectively, are listed in Table 1. Calculation of $K_{d,\ put}$ or $K_{d,\ spd}$ from the ratios $k_{-1,\ put}/k_{+1,\ put}$ or $k_{-1,\ spd:index}/k_{+1,\ spd}$ (8.2 or 25.7 μM, respectively, see also Table 1) exhibited a relatively good consistency with K_d values that were determined by equilibrium measurements (K_d = 3.8 or 106 μM, respectively). In the case of SPM, linear regression analysis of the time course of log (100–100 × $[B_t]/[B_{eq}]$) yielded correlation coefficients ≥ 0.93 with ligand concentrations in the 1–10 μM range, but ≤ 0.75 at concentrations higher than 10 μM. The results for SPM concentrations of 1 and 10 μM are illustrated in Fig. 2C. Replots of kinetic data ($k^{*,\ spm}$) obtained with [^{14}C]SPM concentrations of 1, 2.5, 5 or 10 μM (Fig. 2F) gave $k_{+1,\ spm}$ and $k_{-1,\ spm}$ values of 2.5 ± 1.8 s^{-1} M^{-1} and (3.0 ± 1.1) × 10^{-6} s^{-1}, respectively (also shown in Table 1). Moreover, the value of 1.2 μM that was derived from the ratio of the previous $k_{-1,\ spm}$ and $k_{+1,\ spm}$ data agreed reasonably well with the K_d value of 2.5 μM reported above for site spM_1. On the other hand, attempts to replot the slopes of the time courses of SPM binding obtained for concentrations ranging from 1 to 243 μM or from 20 to 243 μM gave a $K_{d,\ spm}$ value ≥ 190 μM (not illustrated) that diverged strongly from both K_d values determined from equilibrium binding studies for sites spM.

As already mentioned, the association of all three radiolabeled polyamines with IBBM was stable for at least 30 hr at 4°. Reversibility was observed at this temperature, but dissociation was very limited in the presence of 10 mM

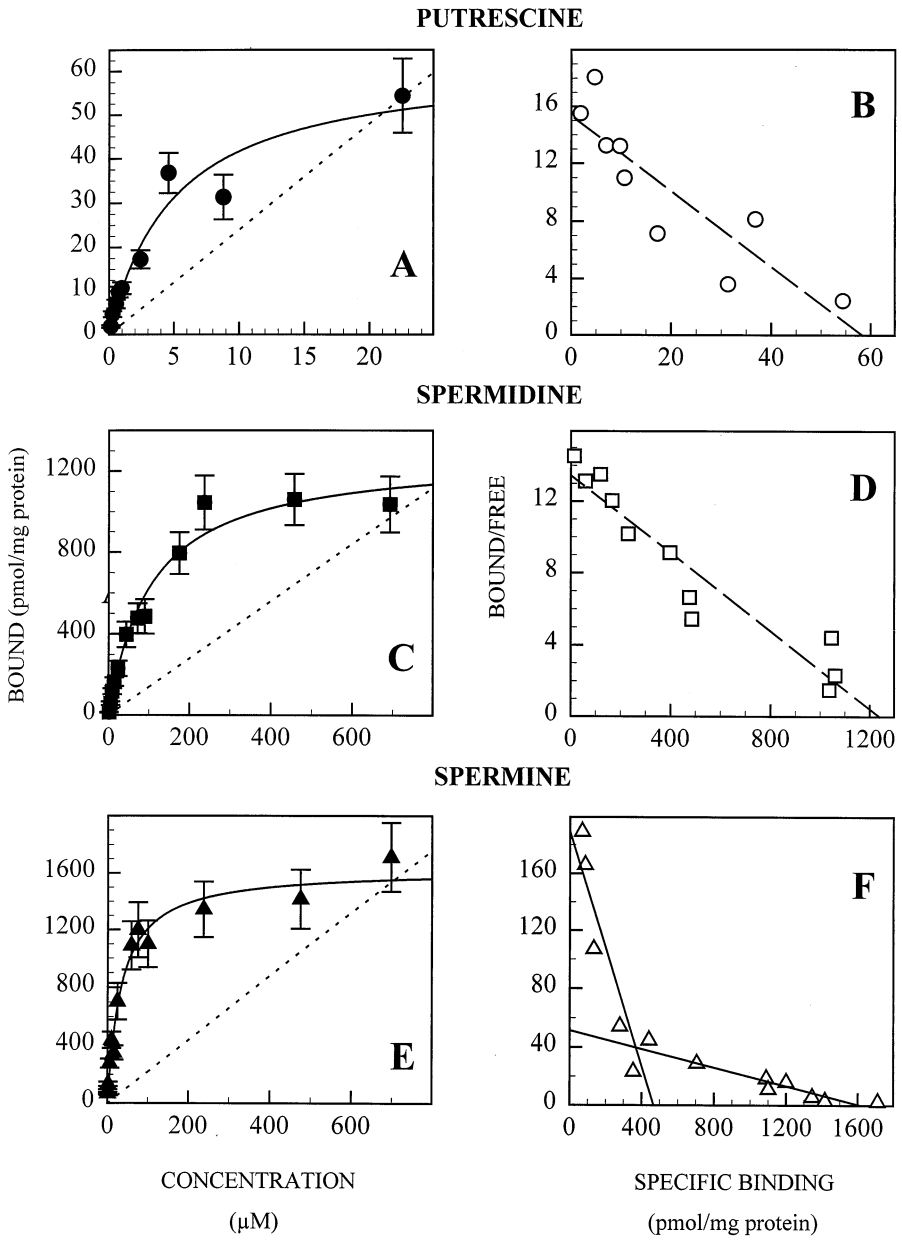

FIG. 1. Equilibrium binding of radiolabeled polyamines to rabbit IBBM. Specific (——) and nonspecific (·····) binding of 0.1–25 μM [^{3}H]PUT (A), 0.1–700 μM [^{3}H]SPD (C) and 0.1–700 μM [^{14}C]SPM (E) were measured as described under Materials and Methods in the absence and presence of 10 mM nonradioactive PUT, SPD or SPM, respectively. ●, ■ and ▲ represent the means of experimental data for specific binding while vertical bars stand for SD. Straight lines (——) represent computer modeling of these data. Dotted lines (·····) result from the fit of nonspecific binding data (not illustrated) to a straight line with a slope of 2.42, 1.39 and 2.21 for PUT, SPD and SPM, respectively (correlation coefficients ≥ 0.98). Each point is the mean value of triplicate measurements with two different membrane suspensions. (B, D and F) Scatchard analyses of specifically bound [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM, respectively. Dashed lines (- - -) result from the fit of experimental data for specific binding of PUT ○ or SPD □ to a straight line with a slope of −0.263 or −0.0108 (correlation coefficients of 0.88 and 0.95), respectively. Straight lines (——) result from the fit of data for specific binding of SPM (Δ) to two straight lines with a slope of −0.406 (correlation coefficient = 0.91) or −0.0319 (correlation coefficient = 0.89) obtained at [^{14}C]SPM concentrations of 0.1 to 15.2 and 24.3 to 700 μM, respectively.

unlabeled polyamine. For instance, following an 18-hr preincubation of IBBM with 1 μM [^{3}H]PUT or 49 μM [^{3}H]SPD, less than 40% of the polyamine-membrane complex was dissociated by 48 hr (not illustrated). The slow dissociation rate of polyamines from IBBM at 4° was consistent with half-times of dissociation higher than 50 hr for PUT and SPD, as derived from the $k_{-1,\,put}$ and $k_{-1,\,spd}$ values, respectively, obtained in the above time courses of association. Dissociation was therefore studied at 23°, a temperature at which polyamines are not degraded when incubated with IBBM vesicles [19]. Dissociation of [^{3}H]SPD or [^{14}C]SPM at 23° (Fig. 3B and 3C, respectively) was

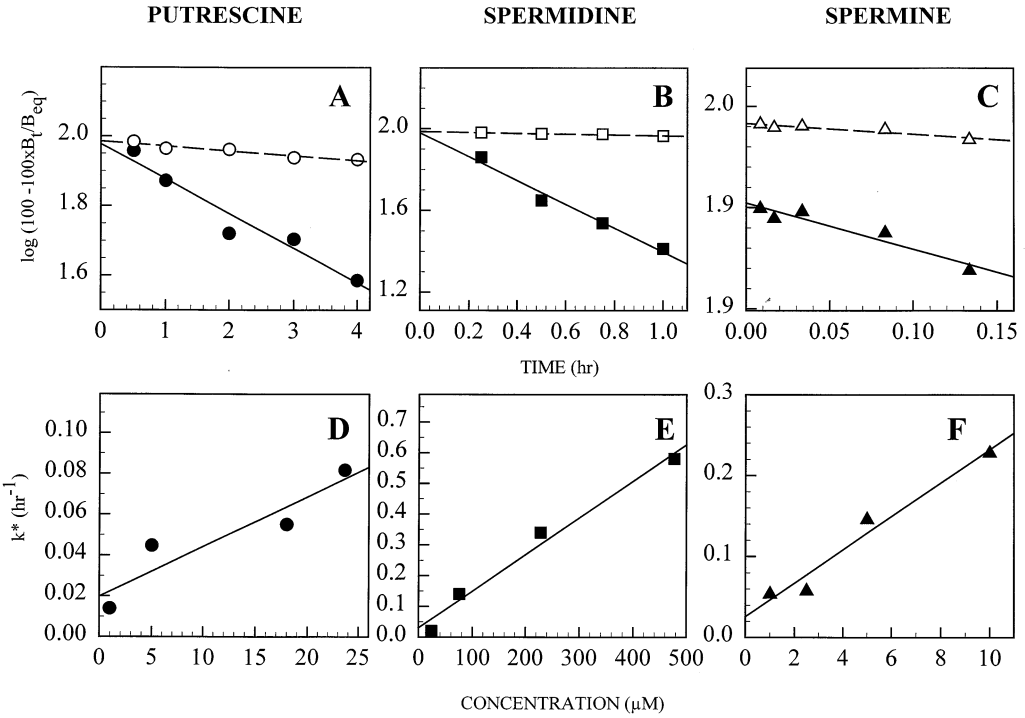

FIG. 2. Association rates of [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM. The time course of association between membranes (0.5 mg/mL) and radioactive polyamine was studied at 4° over a variety of ligand concentrations. (A and B) Semilogarithmic plots of the time courses for association of 1 (○) or 23 μM (●) PUT and 24 (□) or 477 μM (■) SPD, respectively (correlation coefficients ≥ 0.96). (C) The same plot obtained with SPM concentrations of 1 (△) and 10 (▲) μM (correlation coefficients ≥ 0.94). (D, E and F) Secondary plots of the slope of the straight lines (correlation coefficients ≥ 0.93) thus obtained as a function of [^{3}H]PUT (●), [^{3}H]SPD (■) or [^{14}C]SPM (▲) concentrations ranging from 1 to 23 μM, 24 to 477 μM or 1 to 10 μM, respectively. The values of the association and dissociation rate constants $k_{+1,\ put}$; $k_{+1,\ spd}$; $k_{+1,\ spm}$; $k_{-1,\ put}$; $k_{-1,\ spd}$ and $k_{-1,\ spm}$ that were derived from the later plots are listed in Table 1. Data are means of triplicate measurements with two different membrane suspensions.

markedly different from that of [^{3}H]PUT (Fig. 3A), because it could occur only after addition of 10 mM unlabeled SPD or SPM. This was in sharp contrast with [^{3}H]PUT dissociation that took place merely after transfer of the radiolabeled ligand-membrane complex from 4° to 23°. However, PUT dissociation could be accelerated in the presence of 10 mM unlabeled diamine. The semilogarithmic plots of the time courses of dissociation of [^{3}H]SPD (Fig. 3B), unlike [^{14}C]SPM (Fig. 3C), could be satisfactorily fitted to a straight line (correlation coefficient = 0.95 vs 0.63). As shown in Fig. 3A, first-order dissociation was also observed in the case of PUT, with or without addition of unlabeled diamine (correlation coefficients ≥ 0.95). On the other hand, [^{14}C]SPM dissociation was best fitted to a biphasic model that could be split into an early fast phase and a late slow phase (correlation coefficients ≥ 0.85). The values for the half-times of dissociation of all three polyamines that were derived from Fig. 3 are listed in Table 2.

Specificity of Polyamine Binding

As shown in Fig. 4, [^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM were displaced from their binding site(s) by increasing concentrations of nonradioactive polyamines or polyamine ana-

TABLE 1. Association and dissociation rate constants of [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM at 4°

	Ligand		
Concentration range	**[^{3}H]PUT 1–25 μM**	**[^{3}H]SPD 24–477 μM**	**[^{14}C]SPM 1–10 μM**
Association rate constant, k_{+1}, s^{-1} M^{-1}	0.29 ± 0.14	0.14 ± 0.05	2.5 ± 1.8
Dissociation rate constant, k_{-1},$s^{-1} \times 10^6$	2.4 ± 1.0	3.7 ± 2.5	3.0 ± 1.1
Calculated equilibrium dissociation constant, $K_{d,\ calc}$, μM	8.2	25.7	1.2

Measurements were carried out at 4° with the indicated polyamine concentrations, as described in the legend to Fig. 2. The association ($k_{+1,\ put}$; $k_{+1,\ spd}$ and $k_{+1,\ spm}$) and dissociation rate constant values ($k_{-1,\ put}$; $k_{-1,\ spd}$ and $k_{-1,\ spm}$) were derived from the slope and y-intercept of the replots shown in Figs. 2D, 2E and 2F for binding of [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM, respectively. $K_{d,\ calc,}$ represents the equilibrium dissociation constant calculated form the ratio of the k_{-1} and k_{+1} values determined for each polamine as indicated above. Data are mean values of triplicate measurements with two different membrane suspensions.

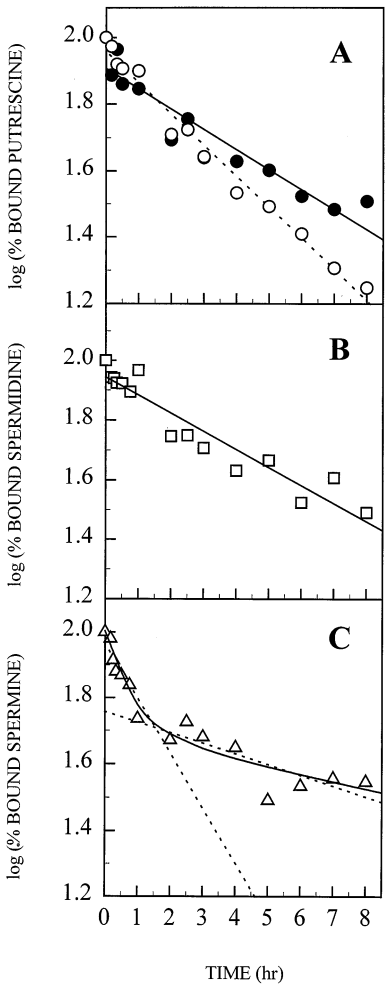

FIG. 3. [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM dissociation rates. Time courses of dissociation were measured by preincubating IBBM (0.5 mg/mL) for 18 hr at 4° with one radiolabeled polyamine and transferring the membranes to a 23° water bath after addition of a one-hundredth volume of distilled water or 1 M stock solution of the corresponding unlabeled polyamine. (A) Semilogarithmic plots of PUT dissociation with (○···○) or without (●——●) addition of unlabeled PUT to IBBM preloaded with 0.95 μM [^{3}H]PUT; (B and C), semilogarithmic plots of dissociation of SPD (□——□) or SPM (△——△) from membranes preloaded with 49 μM [^{3}H]SPD or 22 μM [^{14}C]SPM, respectively. Data are differences between matched determinations ± excess unlabeled polyamine. All data are means of triplicate measurements with two different membrane suspensions. For [^{3}H]PUT (○ and ●) and [^{3}H]SPD (□) dissociation, the plots were best fitted to a straight line model (coefficient correlations ≥ 0.95). On the other hand, the plot for [^{14}C]SPM dissociation was curvilinear (coefficient correlation = 0.63) and could be split into two straight lines (coefficient correlations = 0.94 and 0.85). The dissociation rate constant values, $^{23}k_{-1}$, measured at 23° and derived from the slope of each line, are given in Table 2.

logs. However, the features of PUT displacement, on the one hand, and SPD or SPM displacement, on the other, differed strikingly. PUT binding was dramatically sensitive to MGBG (Fig. 4B), a potent inhibitor of the polyamine biosynthetic enzyme S-AMDC [2, 35, 36] and of polyamine transport [3, 9, 20]. This was in sharp contrast to the moderate inhibitory effect of SPD and SPM (Fig. 4A). Saturation isotherms of [^{3}H]PUT binding in the presence of different concentrations of MGBG were determined. Scatchard analysis of the data revealed that the drug acted as a competitive/noncompetitive inhibitor since, in contrast to SPD and SPM, it decreased not only K_d but also B_{max} for PUT binding (not illustrated). [^{3}H]PUT displacement by CAD (Fig. 4C) resembled that caused by PUT itself (result not illustrated). Additionally, [^{3}H]SPD binding (Fig. 4D and 4E) was either poorly affected or not affected at all by PUT, MGBG or CAD in contrast with SPM. Bound [^{14}C]SPM (Fig. 4F and 4G) could not be displaced by the two diamines and was minimally affected by MGBG as well. The inhibition curve for [^{14}C]SPM by SPD was biphasic with first a decrease in the 50–200 μM SPD concentration range, then a plateau, and finally a second decreasing phase at concentrations higher than 600 μM.

The K_i values of the above inhibitors determined by Log-Logit analysis using the computer program EBDA-LIGAND [34] are summarized in Table 3. The result (K_i in the nanomolar range vs micromolar range) underlines the high potency of MGBG compared to other inhibitors to specifically displace [^{3}H]PUT from its binding site. It also points out the similarity between the K_i value (42 μM) for inhibition of [^{3}H]SPD binding by SPM and the K_d value (31 μM) determined for [^{14}C]SPM binding to site spM_2. Moreover, the K_i value (115 μM) that was derived from the displacement curve of [^{14}C]SPM by SPD in the 50–200 μM concentration range agreed well with the $K_{d,\ spd}$ value (106 μM) determined from binding studies. On the other hand, the K_i value measured for inhibition of [^{3}H]SPD binding by PUT (211 μM) differed greatly from the $K_{d,\ put}$ value (3.8 μM). Finally, roughly similar K_i values (540 and 684 μM) were observed for inhibition of [^{3}H]SPD and [^{14}C]SPM, respectively, by MGBG.

DISCUSSION

The current paper characterizes different sites in plasma membranes from rabbit small intestine that are specific to polyamine binding. From the determination of saturation isotherms, we identified: 1) a high-affinity/low-density site we called puT that binds the polyamine precursor, the diamine PUT, its C_5 derivative CAD and especially the antileukemic agent MGBG, a potent inhibitor of mammalian S-AMDC; 2) a low-affinity/high-density site we called spD that binds SPD; and 3) two sites we called spM_1 and spM_2 that exhibit characteristics of high affinity/low density and low affinity/high density, respectively, for SPM binding.

The K_d value for specific binding of PUT that has been determined in the current study is of the same order of magnitude as the $K_m$values previously reported for uptake of PUT by IBBM vesicles (16.8 and 23 μM [19] and 12.7 μM [20]) or polarized intestinal cell cultures (4.9 μM [18] and 0.41 μM [37]). Additionally, all the ligands we found able

TABLE 2. Dissociation rate constants of polyamines at 23°

	Ligand			
	[^{3}H]PUT	[^{3}H]SPD	[^{14}C]SPM	
			Site 1	Site 2
Dissociation rate constant, $^{23}k_{-1}$,$s^{-1} \times 10^6$	11.3 ± 5.0	7.3 ± 4.2	20.0 ± 7.5	3.9 ± 2.1
Half time of dissociation, $^{23}t^{1/2}$, hr	17.1	26.4	9.6	49.9

Membranes were preincubated for 18 hr at 4° with 0.95 μM [^{3}H]PUT, 49 μM [^{3}H]SPD or 22 μM [^{14}C]SPM, and dissociation was measured at 23° by adding 10 mM of the corresponding unlabeled polyamine. The dissociation rate constant values of the three polyamines at 23° ($^{23}k_{-1,}$) were derived from the slope of the straight lines depicted in Figs. 3A, 3B and 3C. Their half-time of dissociation, $^{23}t^{1/2}$, was calculated from the ratio $0.693/^{23}k_{-1,}$. Note that $^{23}k_{-1,put}$ and $^{23}t^{1/2,put}$ without addition of 10 mM unlabeled PUT to the dissociation medium were 7.2×10^{-6} s^{-1} and 26.4 hr, respectively. Data are mean values of triplicate measurements with two different membrane suspensions.

to prevent PUT from binding to site puT have previously been shown to inhibit its uptake by IBBM vesicles [19, 20]. More particularly, SPD and SPM were less efficient competitors of both transport and binding of [^{3}H]PUT than CAD and PUT itself. Quantitative differences, however, were observed between the potency of SPD and SPM as displacers of PUT binding (see K_i data in Table 3) and their ability to compete with PUT for uptake (K_i = 117 and 219 μM for SPD and SPM, respectively [19]). Compared with polyamines, MGBG was an extremely potent inhibitor of PUT binding to site puT, with a K_i value in the nanomolar range and total inhibition achieved at micromolar concentrations. The result is comparable to that (IC_{50} = 32 nM) obtained for inhibition of PUT transport by MGBG in human pulmonary artery endothelial cells [38]. Regarding intestinal uptake of PUT, although inhibition by MGBG has previously been reported [19, 20], no attempts have been made to evaluate the precise K_i of the drug. An IC_{50} value far below 10 μM is suggested by the data published by Milovic *et al.* [20] from inhibition experiments of PUT transport by MGBG in IBBM vesicles. The mechanism whereby MGBG inhibited PUT uptake is still unclear. Scatchard analysis of [^{3}H]PUT binding in the presence of various concentrations of MGBG revealed that the drug acted as a mixed competitive/noncompetitive inhibitor, in contrast to SPD and SPM that acted in a purely competitive manner (not shown). The two highly basic terminal guanidinium groups of MGBG might be responsible for its markedly strong and/or noncompetitive effect(s) on PUT binding. The effect of the analogue may be explained by the existence on the PUT transporter of two interacting MGBG binding sites, one of which might be site puT. Nevertheless, such an effect may also be indirect [39] and our findings reflect mechanisms such as alterations in the transmembrane gradient of pH or electrical potential or in the availability of sites for PUT binding. In studies on the effect of polyamine transport on membrane potential using fluorescence of the cyanine dye diS-C_2- [5] as a marker [40], we observed that MGBG caused hyperpolarization of the electrical potential difference across rabbit IBBM. This was in sharp contrast to polyamines which increased fluorescence in a concentration-dependent manner, suggesting that membrane depolarization was due to a net flow of positive current (Brachet P, Hargittai PT, Terrian DM and Seidel ER, unpublished observations). However, noncompetitive inhibition of PUT binding by MGBG does not seem to be due to nonspecific alterations of the membranes, since the drug exhibited a weak effect on SPD and SPM binding (see Table 3). Further studies are needed to delineate the mechanisms of action of MGBG on PUT binding (and transport) in IBBM vesicles. This additionally will help to determine whether MGBG as such and less cytotoxic analogs may be useful in specifically blocking uptake of exogenous polyamines by the gut, and consequently at reducing polyamine supply to tumors [41].

In vivo studies have reported that 23–47% of radioactivity was cleared from the small intestine within 1 hr of administration of [^{14}C]polyamines to rats by intragastric intubation [42]. These studies and others on PUT transport across polarized intestinal epithelial cells [17, 18] clearly suggested that bound polyamines may readily be released from their binding sites on the IBBM *in vivo*. Our present data, however, indicate that this release is strongly dependent upon temperature since first, we measured half-times of dissociation ranging between 9.6 and 49.9 hr at 23° and second, polyamine association with the membranes was stable for at least 30 hr at 4°. The very slow dissociation rates of radioactive polyamines at ambient temperature make them valuable ligands for specific labeling and further identification of sites puT, spD, spM_1 and spM_2. In contrast to [^{3}H]SPD and [^{14}C]SPM, [^{3}H]PUT could dissociate from its binding site at 23° without addition of unlabeled diamine. Although we do not have any direct evidence thus far, PUT binding to site puT may be sensitive to alterations in the fluidity of the membrane bilayer that accompany the temperature shift from 4 to 23°. Such alterations might be responsible for conformational changes in site puT, weakening binding of PUT and finally leading to its dissociation. Moreover, we previously observed that efflux of [^{3}H]PUT taken up by rabbit IBBM vesicles could occur at 25° merely by diluting the membranes with an isotonic buffer [19]. This common sensitivity to physicochemical changes, in addition to the above-mentioned overall good correlation between the inhibition patterns of ligands, supports the contention that site puT and PUT transporter may belong to the same entity.

Both measurements of saturation isotherms and association/dissociation kinetics favor the existence of two sites

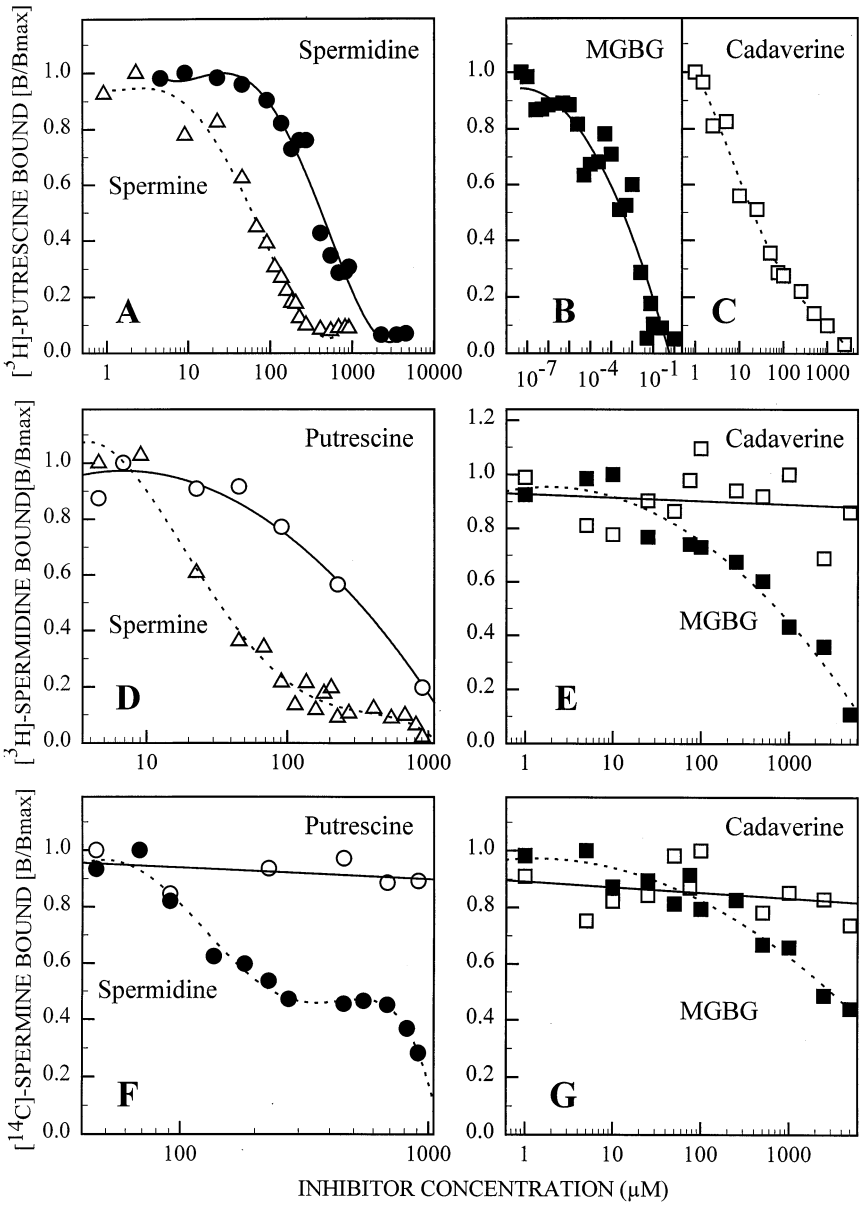

FIG. 4. Displacement of bound [^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM by other polyamines and analogs. Membranes (0.5 mg/mL) were incubated for 18 hr at 4° in the presence of 0.95 μM [^{3}H]PUT (A, B and C), 49 μM [^{3}H]SPD (D and E) or 23 μM [^{14}C]SPM (F and G) plus the indicated concentrations of unlabeled PUT (○), SPD (●), SPM (△), CAD (□) or MGBG (■). Bound radioactivity (means of triplicate measurements with two different membrane preparations) is plotted against the concentration of effector. Data were determined by Log-Logit analysis using the computer program EBDA-LIGAND as stated under Materials and Methods. The curves illustrate computer modeling of results. The values for the inhibition constants, K_i, obtained for SPD, SPM, CAD and MGBG are listed in Table 3.

and a single site of binding for SPM and SPD, respectively. Additionally, the displacement curve of bound [^{14}C]SPM by SPD (see Fig. 4F) displayed a profile with a shallow steepness that suggests a greater complexity of SPM-membrane interactions than accounted for by binding of this ligand to a single population of sites. Moreover, because B_{max} values of SPD and SPM binding to sites spD and spM_2 were not significantly different (1240 ± 160 and 1617 ± 247 pmol/mg of protein, respectively), both sites might be identical. This assumption is strengthened by the findings that similar inhibition features for binding of both polyamines were observed with all the ligands tested (see Table 3). MGBG, despite the resemblance of its chemical structure to that of SPD or SPM, only weakly affected binding of the two polyamines to IBBM. A poor inhibition or no alteration at all of SPD or SPM binding was also caused by both diamines, PUT and CAD. Our results indicate that there may be a multiplicity of sites that specifically bind polyamines in IBBM. Interaction of PUT and SPD (or SPM) with separate transport systems has previously been

TABLE 3. Inhibition of [^{3}H]PUT, [^{3}H]SPD and [^{14}C]SPM binding by polyamines and analogs

Inhibitor	Ligand		
	[^{3}H]PUT	[^{3}H]SPD	[^{14}C]SPM
PUT	—	211 ± 34	NM*
SPD	325 ± 51	—	115 ± 26
SPM	47 ± 10	42 ± 11	—
CAD	2.3 ± 0.4	NM*	NM*
MGBG	0.0032 ± 0.0015	540 ± 162	648 ± 143

Inhibition constants (K_i) of compounds able to displace [^{3}H]PUT, [^{3}H]SPD or [^{14}C]SPM from binding sites on IBBM were determined as described in the legend to Fig. 4. Data represent means ± SD of triplicate measurements with two membrane preparations. *NM: not measurable.

found in rat duodenal crypt IEC-6 cells [18], isolated rat enterocytes [27], Chinese hamster ovary cells [28], B16 melanoma cells [30], human endothelial cells [43] or pancreatic acinar tumor cells [44]. More particularly, in many of the previous models, PUT exhibited no or a weak inhibitory effect on SPD or SPM uptake, while its uptake was inhibited by the two larger polyamines. Although some convergences therefore exist between previous transport data and our current observations, there is no strong evidence for a link between sites spD, spM_1 or spM_2 and any polyamine transport system kinetically described. Polyamines have been shown to interact with membrane components other than their transport systems. For instance, multiple recognition sites for polyamines have been suggested for the *N*-methyl-D-aspartate receptors to account for their complex modulatory activity or for inward rectification of a class of K^+ channels [45, 46]. Further investigations have to be carried out to ascertain the relevance of the different specific binding sites of polyamines evidenced herein to their transport systems.

In summary, we have characterized multiple sites for specific binding of polyamines to the IBBM. Several lines of evidence obtained from saturation isotherms, kinetics of association and dissociation, and competition experiments suggest that, unlike PUT and SPD, SPM may preferentially bind two separate types of sites. These sites appear to exhibit a ligand selectivity, notably with respect to diamines and MGBG, that is markedly different from that of the PUT binding site. Evidence is also given herein that the latter might be associated with the transport system already described for the intestinal uptake of PUT. Due to the high stability of binding of polyamines at 4°, the current data may form the basis of a discriminatory approach to their transport systems.

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