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European Journal of Pharmacology 403 (2000) 9–15

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Study of serotonin interactions with brush border membrane of rabbit jejunum enterocytes

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Received 17 February 2000; received in revised form 3 July 2000; accepted 7 July 2000

Abstract

Recent studies have demonstrated that serotonin (5-hydroxytryptamine, 5-HT) may interact with either specific receptors or with a specific transporter that takes up 5-HT in the gastrointestinal tract. The purpose of the present work was to study the 5-HT interactions with brush border membrane from rabbit jejunum enterocytes. The results obtained showed that 5-HT did not seem to be transported by any specific system of transport in brush border membrane vesicles. Nevertheless, [^{3}H]5-HT seemed to bind specifically to this membrane. The kinetic analysis indicated a saturable and dissociable specific binding with a dissociation constant $K_D = 14 \times 10^{-9}$ M. The saturation studies with [^{3}H]5-HT indicated the presence of one specific site in the brush border membrane. The results of displacement of [^{3}H]5-HT specific binding from the brush border membrane showed that both unlabelled 5-HT and unlabelled GR113080 ([1-[(2-methyl sulphonyl) amino] ethyl-4-piperidinyl] methyl-1-methyl-1*H*-indole-3-carboxylate), a specific competitive antagonist of 5-HT$_4$ receptors, inhibited the specific binding of [^{3}H]5-HT to this membrane.

Keywords: 5-HT (5-hydroxytryptamine, serotonin) transport; 5-HT receptor; Brush border membrane; Jejunum, rabbit

1. Introduction

5-Hydroxytryptamine (5-HT) is stored in granular vesicles in enterochromaffin cells and in nerves of the gastrointestinal tract. 5-HT plays an important role in the regulation of gastrointestinal physiology, either acting as a transmitter of interneurons in the mesenteric plexus (Costa and Furness, 1979; Wade et al., 1994) or being released from intestinal stores by drugs or vagal stimulation (Schwörer et al., 1992). Cloning studies have revealed the existence of at least seven 5-HT receptors subdivided into 14 subtypes (Lucas and Hen, 1995), most of them being present in the gastrointestinal tract. Thus, 5-HT receptors are involved in the modulation of 5-HT release from enterochromaffin cells (Gebauer et al., 1993). 5-HT has been shown to affect gastrointestinal motility by acting through 5-HT receptors in the enteric nervous system (Gershon, 1995; Foxx-Orenstein et al., 1996) and musculature (Engel et al., 1984; Pinkus et al., 1989; Kuemmerle et al., 1992), and to alter intestinal electrolyte transport (Sundaram et al., 1991; Franks et al., 1996) in which 5-HT receptors of the mucosa or neural elements are involved (Beubler and Horina, 1990; Imada-Shirakata et al., 1997; Borman and Burleigh, 1997; McLean and Coupar, 1998). In addition, 5-HT has also been shown to inhibit the intestinal absorption of sugars (Arruebo et al., 1989) and L-leucine (Salvador et al., 1996) by acting through several 5-HT receptors (Salvador et al., 1997) and involving intracellular processes.

Although many studies have been carried out to determine the physiological role of 5-HT in the gastrointestinal tract and the receptors involved, recent results have determined the mechanism by which 5-HT is inactivated in order to avoid desensitization of these receptors. Thus, the presence of a 5-HT transporter has been described in rat jejunum (Takayanagi et al., 1993; Wade et al., 1996) and in guinea pig intestine (Chen et al., 1998). This transporter might take up 5-HT to inactivate it.

The specific location of the 5-HT receptors involved in the 5-HT inhibition of nutrient absorption in rabbit je-

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00484-2

junum remains unknown and, furthermore, the presence of a 5-HT-specific system of transport in the rabbit jejunum enterocytes cannot be discarded. Thus, the purpose of this work was to study, firstly, the presence of 5-HT transport systems in the brush border membrane vesicles of the rabbit jejunum enterocyte and, secondly, the specific binding of 5-HT to the brush border membrane of the enterocyte from rabbit jejunum.

2. Materials and methods

2.1. Animals

Animals were handled according to the European Council Legislation 86/609/EEC on experimental animal protection. Two-month-old, male New Zealand rabbits weighing about 1.5–2.0 kg were caged separately in a room kept at a constant temperature (24°C), with free access to water and standard rabbit fodder. The rabbits were killed by a blow on the nape, and the proximal jejunum was rapidly removed, rinsed free of intestinal contents with ice-cold Ringer solution containing 0.1 mM of phenylmethylsulfonyl fluoride.

2.2. Brush border membrane vesicles preparation

Brush border membranes were prepared from the jejunum by the Mg^{2+} precipitation method of Kessler et al. (1978) modified by Hauser et al. (1980). The whole procedure was carried out at 4°C. Briefly, the mucosal scrapings of the jejunum were suspended in 2 ml/g of buffer I containing 10 mM HEPES–Tris, pH 7.4 and 300 mM D-mannitol, supplemented with 5 mM EGTA plus 0.1 mM phenylmethylsulphonyl fluoride. The suspension was homogenized by using fifteen 5-s bursts of an Ultra-turax adjusted to 50% of its maximal speed. The homogenate was diluted by one-half with buffer II: 10 mM HEPES–Tris pH 7.4 and 700 mM D-mannitol (a sample was taken to measure enzymatic activity) and was treated with 10 mM Mg^{2+}. The resulting suspension was centrifuged for 15 min at $3000 \times g$. The pellet P_1 was discarded and the supernatant S_1 was centrifuged for 40 min at $30{,}000 \times g$. The new pellet P_2 was resuspended with buffer III: 10 mM HEPES–Tris pH 7.4 and mannitol 500 mM and was homogenized with nine strokes of a glass Teflon homogenizer. The homogenate was treated again with 10 mM Mg^{2+} and centrifuged for 15 min at $3000 \times g$. The supernatant (S_3) was then centrifuged for 40 min at $30{,}000 \times g$. The pellet (P_4 = brush border membrane) was suspended with buffer III up to 20–25 mg protein/ml and was immediately placed on ice to be used in the experiments. Protein was measured with the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The purity of the brush border membrane preparation was determined by the measurement of either sucrase enzymatic activity, using the method of Dahlqvist (1964), or Na^+–K^+-ATPase activity (Proverbio and Del Castillo, 1981), in this case to calculate the possible contamination of the brush border membrane with basolateral membrane. The brush border membrane vesicles were enriched 19 times and the contamination with basolateral membrane was 3%. Furthermore, previous to the use of the brush border membrane vesicles, the uptake of D-glucose 0.1 mM at different times (5, 10, 40, 60 s and 2 h) and at 25°C was determined in order to assure the functioning of the brush border membrane vesicles.

2.3. 5-HT transport in brush border membrane vesicles

5-HT transport across brush border membrane vesicles was assayed by using a rapid filtration technique with 5-HT as the substrate plus [^{3}H]5-HT as a radio tracer at different concentrations depending on the experiment. Two different experiments were carried out: (1) 5-HT 0.1 μM uptake was measured at different times (5, 10, 40, 60 s and 2 h) (time course uptake) and (2) a kinetic study in which 5-HT uptake was measured at different concentrations ranging from 0.05 to 10 μM and the time of incubation was 10 s. These results were analyzed by non-linear regression with an iMac version of GraphPad Prism 2.0 (GraphPad, San Diego, CA) and the kinetic constants K_m and V_{max} were calculated. In both experiments, the incubation temperature was 25°C and the intravesicular buffer solution contained 500 mM D-mannitol and 10 mM HEPES–Tris pH 7.4. The incubation solution was prepared by adding the different substances (NaCl, 5-HT and imipramine) and the osmolarity was maintained constant at 500 mosM/l with D-mannitol. The uptake was stopped by adding stop solution containing in mM 10 HEPES–Tris pH 7.4; 350 KCl, 25 $MgSO_4$. The radioactivity was measured, after adding scintillation liquid, in a Beta Counter. The time course and kinetic experiments were carried out under three different conditions: (i) in the presence of a Na^+ gradient ($[Na^+]in/[Na^+]_{out} = 0/100$ mM); (ii) in the absence of a Na^+ gradient, with Na^+ being replaced by choline$^+$; (iii) in the presence of Na^+ gradient and imipramine (0.1 mM).

2.4. Binding of 5-HT

Binding of 5-HT to brush border membranes was determined by modifying the procedures previously described by Dharmsathaphorn et al. (1983) and Alcalde et al. (1996) using [^{3}H]5-HT as the labelled molecule.

Brush border membrane vesicles were suspended in a buffer solution containing 10 mM HEPES–Tris pH 7.4, 10 mM $MgCl_2$. This solution was hyposmotic in order to disrupt the vesicles. The total volume of the reaction mixture (100 μl) contained 1 mg protein/ml brush border membrane vesicles and [^{3}H]5-HT at different concentrations depending on the experiment. Non-specific binding

was measured in the presence of 10 μM unlabelled 5-HT in parallel incubation tubes. The [^{3}H]5-HT specific binding was calculated by subtracting the [^{3}H]5-HT non-specific binding from the total binding. The nonspecific binding was 45%–50% of the total binding. The experiments were performed at 20°C for 10 min. These experimental conditions were chosen after preliminary binding determinations. The incubations were carried out with constant shaking.

The reaction was stopped by adding 3 ml of ice-cold buffer solution (see above) and the mixtures were then immediately filtered through nitrocellulose filters which were prewetted with the buffer solution containing 5-HT 1 μM. The filters were washed twice with 3 ml of ice-cold buffer solution, and the radioactivity was measured, after adding scintillation liquid, in a Beta Counter. In the experiments for kinetic analysis of the [^{3}H]5-HT specific binding and calculation of the association-dissociation rate constant, the second-order rate constant for association (k_1) was calculated with the equation: $k_1 = (k_{ob} - k_{-1})/[^3\text{H-5-HT}]$, where [^{3}H-5-HT] was 50 nM and the kinetically derived dissociation constant (K_D) was calculated from the ratio k_{-1}/k_1. Binding data were analyzed by computer-assisted non-linear regression analysis (Prism version 2.0 for iMac computer).

2.5. Drugs

5-HT and D-glucose were purchased from Sigma (St. Louis, MO, USA). [^{3}H]5-HT and [^{14}C]D-glucose were obtained from Amersham (International Amersham, UK). LY53857 (6-Methyl-1-(1-methylethyl)ergoline-8β-carboxilic acid 2-hydroxy-1-methylpropyl ester maleate) was obtained from Research Biochemicals (Natick, MA, USA). GR113808 ([1-[(2-methyl sulphonyl) amino] ethyl-4-piperidinyl] methyl-1-methyl-1*H*-indole-3-carboxylate), maleate salt was a gift from Glaxo Welcome (Greenford, England). Ondansetron was a gift from Vita (Barcelona, Spain).

2.6. Data analysis

Where relevant, data are expressed as the means ± S.E.M. and the statistical comparisons were performed by one-way analysis of variance (ANOVA). Data were analyzed by computer-assisted Prism GraphPad Program (Prism version 2.0 for iMac computer).

3. Results

3.1. 5-HT transport in brush border membrane vesicles

5-HT uptake was measured at different times either in the presence of a Na^+ gradient (control), in the absence of a Na^+ gradient (Na^+ was replaced by choline$^+$) or in the presence of the 5-HT transport inhibitor imipramine 0.1 mM. 5-HT concentration was 0.1 μM according to the value of the Michaelis constant ($K_m = 0.6$ μM) described for 5-HT transport in guinea pig intestine (Chen et al., 1998). The data obtained under the different conditions were compared by one-way ANOVA. This analysis indicated that the values obtained in the absence of a Na^+ gradient or in the presence of imipramine were not significantly different from control values. Consequently, the 5-HT uptake seems not to be dependent on the Na^+ gradient and it seems not to be affected by the presence of imipramine (Fig. 1).

The kinetic study was carried out in order to determine whether 5-HT uptake was saturable and, if so, to calculate the kinetic constants in the presence or absence of a Na^+ gradient and in the presence of imipramine (0.1 mM). The 5-HT concentrations ranged from 0.05 to 10 μM. The results were analyzed by non-linear regression with GraphPad Prism 2.0 by weighting the values by $1/Y^2$ and by using stricter criteria for convergency. The results showed that 5-HT uptake was not saturable, and that the data were consistent with a diffusion process. Furthermore, the absence of a Na^+ gradient or the addition of imipramine to the incubation media did not affect the uptake of 5-HT (Fig. 2). The diffusion constants (K_d) were calculated as 73.88 ± 6.88, 60.62 ± 3.37 and 66.45 ± 2.41 nl/mg protein for Na^+ gradient, imipramine and choline chloride (without Na^+ gradient) conditions, respectively.

3.2. Study of the binding of [^{3}H]5-HT in the brush border membrane: Kinetic analysis. Association–dissociation rate constant

Specific [^{3}H]5-HT binding was measured as a function of time (association), and this binding was reversed by

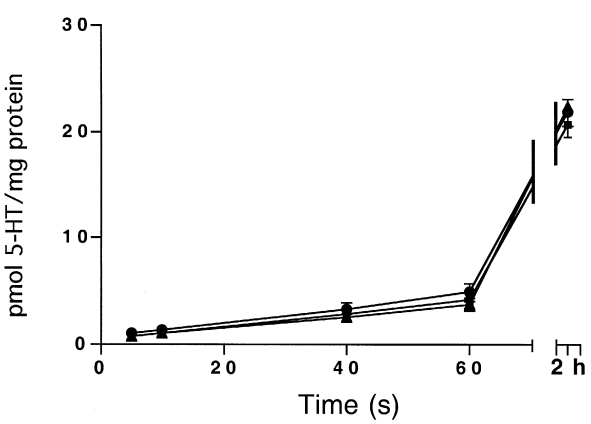

Fig. 1. Study of 5-HT uptake at different times of incubation in brush border membrane vesicles of rabbit jejunum. The incubation was carried out at 25°C. The experimental conditions were control (squared) = in the presence of Na^+-gradient; imipramine (triangle) = imipramine 0.1 mM was added to the medium; choline cloride (circle) = Na^+ was replaced by choline chloride at the same concentration. The results are the means ± S.E.M. of four experiments.

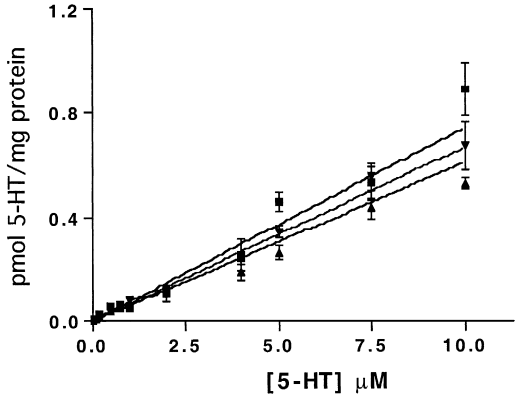

Fig. 2. Kinetic study of 5-HT uptake in brush border membrane vesicles of rabbit jejunum. The incubation was carried out at 25°C and for 10 s. The experimental conditions were control (squared) = in the presence of Na^+ gradient; imipramine (triangle) = imipramine 0.1 mM was added to the medium; choline cloride (circle) = NaCl was replaced by choline chloride at the same concentration. The results are the means ± S.E.M. of four experiments.

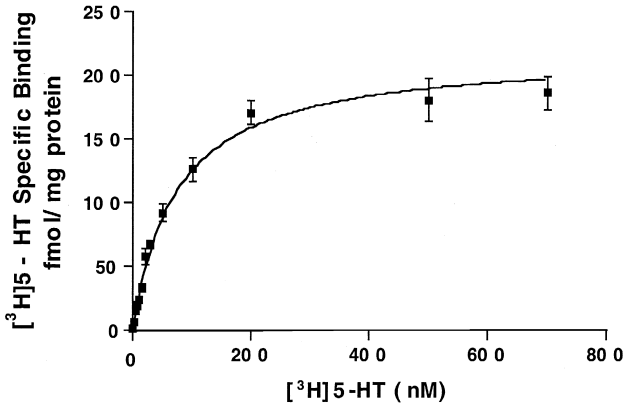

Fig. 4. Saturation study of [^{3}H]5-HT specific binding in the brush border membrane of rabbit jejunum enterocytes. Membranes were incubated 10 min at 20°C with different concentrations of [^{3}H]5-HT. Membrane concentration was 1 mg protein/ml. The results are expressed as [^{3}H]5-HT specific binding and are the means ± S.E.M. of six determinations.

adding a large excess of binding buffer (3 ml) (dissociation). [^{3}H]5-HT binding was saturable, and the steady-state was reached within 10 min at 20°C (Fig. 3). The rate constant (k_{ob}) for the pseudo-first-order association kinetic was 0.427 ± 0.009 min^{-1}. The first-order dissociation rate constant (k_{-1}) was 0.098 ± 0.006 min^{-1}. From these values, the second-order rate constant for association (k_1) was calculated as 6.5×10^6 M^{-1} min^{-1}. The kinetically derived dissociation constant (K_D) calculated was 14×10^{-9} M.

3.3. Saturation analysis of [^{3}H]5-HT binding in brush border membranes

This study was carried out by measuring the specific binding of [^{3}H]5-HT at different concentrations. The results obtained show that the specific binding of [^{3}H]5-HT was saturable (Fig. 4) and the analysis showed a curvilinear graph. The best fit determined a one-site binding model. The K_d (dissociation constant in the steady state) and the B_{max} (maximum capacity of binding to receptors) were calculated as $K_d = 73.5 \pm 8.6$ nM and $B_{max} = 217.5 \pm 19.2$ fmol/mg protein. These results were corroborated

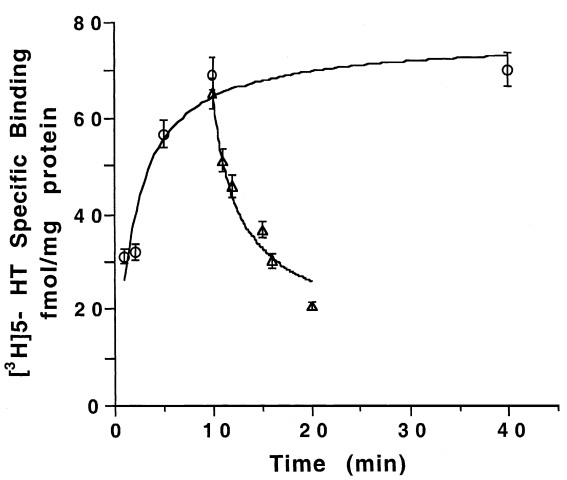

Fig. 3. Association and dissociation kinetics of [^{3}H]5-HT specific binding in the brush border membrane of rabbit jejunum enterocytes. In association experiments (circle), [^{3}H]5-HT (50 nM) was incubated with the brush border membrane at 20°C and at different times. After a 10-min incubation, the dissociation of the specific binding (triangle) was obtained by dilution with 3 ml of buffer 10 mM HEPES–Tris pH 7.4, 10 mM $MgCl_2$, and dissociation was stopped at different times. Membrane concentration was 1 mg protein/ml. The values are the means of four experiments. The results are expressed as the means ± S.E.M.

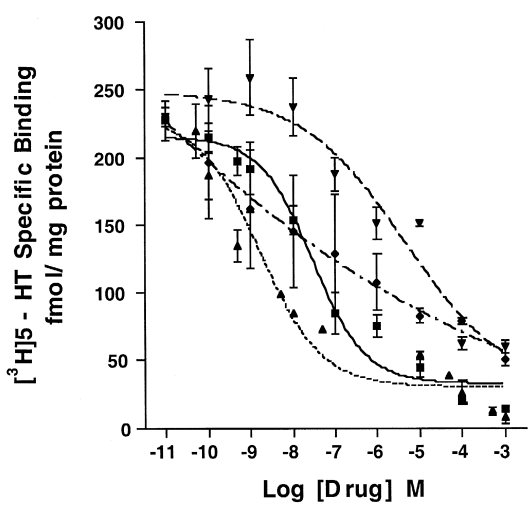

Fig. 5. Displacement study of [^{3}H]5-HT binding to the brush border membrane by increasing concentrations of 5-HT (square) and several unlabelled 5-HT receptor antagonists (5-HT_2 = LY53857 (rhombus); 5-HT_3 = Ondansetron (inverted triangle); 5-HT_4 = GR113080 (triangle)). Time and temperature of incubation were 10 min and 20°C. [^{3}H]5-HT concentration was 500 nM. Membrane concentration was 1 mg protein/ml. The results are the means ± S.E.M. of four experiments.

Table 1
IC_{50}, Hill Slope and K_i values of 5-HT, LY 53857, ondansetron and GR113808A calculated from the competition study of [^{3}H]5-HT specific binding in the brush border membrane

Competing agent	IC_{50} (nM)	K_i (nM)	Hill slope
5-HT	29.70 ± 0.99	3.80	−0.70
GR113808A	1.57 ± 0.60	0.20	−0.60
Ondansetron	4380 ± 2713	643.86	−0.35
LY 53857	4,497,798 ± 543,679	552,361	−0.05

The results are the means of four determinations. Affinity values are given as K_i and were determined from IC_{50} values using the Cheng–Prussoff equation (Cheng and Prussoff, 1973).

by Scatchard analysis (Scatchard, 1949), the values of K_d and B_{max} being similar ($K_d = 69.8$ nM and $B_{max} = 217$ fmol/mg protein).

3.4. Competition studies with brush border membranes

In order to know which 5-HT receptor is present in our brush border membrane preparation, the specific binding of 500 nM [^{3}H]5-HT was measured in the presence of the following unlabelled competitors and antagonists: 5 HT, LY53857 (5-HT2 receptor antagonist), ondansetron (5-HT_3 receptor antagonist) or GR113808 (5-HT_4 receptor antagonist), at different concentrations (ranging from 10 pM to 0.1 mM) (Fig 5).

The results were analyzed by non-linear regression analysis and fitted to a sigmoidal dose–response equation with variable slope. The IC_{50} and Hill Slope were obtained by non-linear regression analysis (Prism version 2.0 for iMac computer) and the K_i values were calculated using the equation of Cheng and Prussoff (1973). Values for IC_{50}, Hill Slope and K_i are given in Table 1.

4. Discussion

The aim of the present work was to determine whether 5-HT interacts with the brush border membrane of rabbit jejunum enterocytes either to be transported or to bind to specific receptors located in this membrane or both. The study of 5-HT transport showed that 5-HT uptake, measured in brush border membrane vesicles, increased linearly with time, but it was not dependent on the Na^+-gradient and it was not inhibited by imipramine, which has been described to be a potent inhibitor of the 5-HT transporter (Chen et al., 1998). Furthermore, the kinetic study of the 5-HT transport in brush border membrane vesicles showed that the results of the uptake did not fit to a saturable equation but to a diffusion equation, and again, neither a gradient of Na^+ nor imipramine affected this transport at any concentration assayed. Consequently, it can be concluded that the brush border membrane of the rabbit jejunum enterocyte is not able to transport 5-HT by a specific and saturable Na^+-dependent system of transport. These results tally with the study of transmural fluxes of 5-HT in guinea pig ileum, which concluded that 5-HT fluxes through this tissue were produced by a passive mechanism (Cooke et al., 1983). Nevertheless, a recent study using in situ hybridization and immunoreactivity to the antibody A-SERT 50 in the epithelium of the guinea pig small intestine has demonstrated the expression of a 5-HT transporter (Chen et al., 1998); however, there is no evidence about the presence of the 5-HT transporter in the brush border membrane. Other authors have concluded that rat isolated enterocytes are able to take up 5-HT and that this transport is saturable and Na^+-dependent (Takayanagi et al., 1993), but they did not exclude the role of the basolateral membrane of the enterocyte.

The results above indicate the absence of a 5-HT transporter in brush border membrane vesicles, but many studies have concluded that 5-HT plays an important role in the physiology of the intestinal epithelium, either on the intestinal absorption of nutrients (Arruebo et al., 1989; Salvador et al., 1996) or on intestinal secretion (Hansen, 1995). Moreover, several authors have concluded that different 5-HT receptors may be involved in these processes (Salvador et al., 1997; McLean and Coupar, 1998). Thus, the second purpose of the present study was to determine the presence of 5-HT-specific receptors in the brush border membrane of rabbit jejunum enterocytes.

The results obtained indicated that [^{3}H]5-HT bound specifically to the brush border membrane and that this binding was saturable. The value of the kinetically derived dissociation constant (K_D) from the association–dissociation experiments (1.4×10^{-8} M) and the K_d (dissociation constant in the steady-state) calculated from the saturation analysis of [^{3}H]5-HT binding (7.3×10^{-8} M) were similar, which seems to indicate the presence of one site in the brush border membrane for binding [^{3}H]5-HT. This observation was confirmed by the evaluation of the Scatchard transformation, which yielded a single binding site ($K_d = 6.9 \times 10^{-8}$ M). B_{max} calculated from the saturation analysis or from the Scatchard transformation was 217 fmol/mg protein.

Since 5-HT_2, 5-HT_3 and 5-HT_4 receptors have been described to be involved either in intestinal secretion (Siriwardena et al., 1993; Kellum et al., 1994; Budhoo and Kellum, 1994; Hansen and Skadhauge, 1997) or in the anti-absorptive effect (Salvador et al., 1997), the interest of the present study was to determine the type of receptor that was present in the brush border membrane. Competition experiments for [^{3}H]5-HT binding to brush border membrane were carried out using 5-HT, LY 53857 (5-HT_2 receptor antagonist), ondansetron (5-HT_3 receptor antagonist) or GR113808 (5-HT_4 receptor antagonist). The results showed that data for 5-HT, ondansetron and GR113808 fitted to a sigmoidal dose–response equation with monophasic displacement curves. The IC_{50} (4380 ± 2713 nM) and K_i (643.86 nM) obtained with ondansetron indi-

cated very low affinity, and these values do not agree with the value published for 5-HT_3 receptors in the rat small intestine ($K_i = 1.3$ nM) (Katayama et al., 1997). Consequently, 5-HT_3 receptors might not be present in the brush border membrane. The results obtained with LY53857 did not fit with competition binding to 5-HT receptors and the values of IC_{50} (0.0045 M), K_i (0.5 mM) and Hill slope (-0.05) indicated that 5-HT_2 receptors might not be present in the brush border membrane of rabbit jejunum enterocytes.

The value of the K_d (dissociation constant in the steady-state) for [^{3}H]5-HT has been calculated in Cos-7 cells expressing 5-HT_4 receptors to be 20 nM, and the affinity of 5-HT in competition with [^{3}H]5-HT binding calculated as K_i was 6.3 nM (Adham et al., 1996). Both values were similar to the constants obtained in the present study ($K_D = 73$ nM and $K_i = 3.8$ nM). Furthermore, the IC_{50} and K_i obtained in our study with GR113808 show that this 5-HT_4 antagonist has a higher affinity for 5-HT_4 receptors in brush border membrane than 5-HT itself. These results tally with previous data using Cos-7 cells transfected with 5-HT_4 receptor cDNA (Blondel et al., 1998), where a [^{3}H]GR113808 K_d value of 0.6 nM was obtained. The shallow competition curves might be due to the possibility that 5-HT_4 receptors have different affinity (Adham et al., 1996; Blondel et al., 1998).

In summary, from the results presented in this study it can be concluded that 5-HT does not seem to be transported by any specific, saturable, Na^+-dependent and imipramine-inhibitable system of transport in the brush border membrane of rabbit jejunum enterocytes. Nevertheless, 5-HT may bind specifically to 5-HT_4 receptors located in this membrane. The presence of 5-HT_2 or 5-HT_3 receptors in the brush border membrane was not demonstrated under our experimental conditions, but this possibility cannot be disregarded.

Acknowledgements

This work has been supported by a grant from Diputación General de Aragon (P CA-2/92) and a grant from Comisión Interministerial de Ciencia y Tecnología (SGPN + D) AGF97-0922. We want to thank Glaxo Wellcome (Greenford, England) for the gift of GR113808A and Vita (Barcelona, Spain) for the gift of Ondansetron.

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