Up-Regulation of P-Glycoprotein Expression in Small Intestine under Chronic Serotonin-Depleted Conditions in Rats

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ABSTRACT

To investigate the role of serotonin (5-HT), an important neurotransmitter and hormone/paracrine agent in the small intestine, in the transport activity of P-glycoprotein (P-gp), the intestinal transport of quinidine, a P-gp substrate, was examined in 5-HT-depleted rats prepared by intraperitoneal administration of p-chlorophenylalanine, a specific inhibitor of tryptophan hydroxylase in 5-HT biosynthesis. In the in vitro transport study, quinidine transport across rat jejunum was significantly enhanced in both the secretory and absorptive directions under 5-HT-depleted conditions, although the secretory transport was still predominant. The electrophysiological study suggested that the quinidine transport via passive diffusion was enhanced presumably through a paracellular route. This might be due to looser tight junctions under 5-HT-depleted conditions. The voltage-clamp technique clearly indicated that the secretory transport of quinidine through the transcellular pathway was also enhanced by the depletion of 5-HT. Furthermore, 5-HT depletion increased verapamil-sensitive secretory transport of quinidine in rat jejunum. These results indicate that the secretory transport of quinidine via P-gp was significantly enhanced under 5-HT-depleted conditions. The level of ATP, an energy source for functioning P-gp, wet weight of jejunum, and total protein level in rat jejunal mucosa were not changed by 5-HT depletion, but the expression of P-gp in the brush-border membrane of rat jejunum was significantly induced, which is partly responsible for the enhancement of P-gp activity under the 5-HT-depleted condition.

P-glycoprotein (P-gp) encoded by the MDR1 gene in humans and mdr1a and mdr1b genes in rodents is a 170-kDa phosphoglycoprotein (Stouch and Gudmundsson, 2002). A topological model based on a hydropathy analysis of the primary sequence indicated two highly homologous halves, each containing six transmembrane helices and one nucleotide-binding domain (Sparreboom et al., 1997). Although P-gp was initially discovered in mammalian tumor cells as one of the causes of multidrug resistance in cancer cells (Sparreboom et al., 1997), this protein has since been identified in many normal tissues including brain, small and large intestines, liver, and kidney, consistent with their role in general detoxification (Kondratov et al., 2001; Stouch and Gudmundsson, 2002). Particularly, P-gp-expressing cells in the small intestine secrete many drugs from the epithelial cells to the lumen, leading to low bioavailability (Sparreboom et al., 1997). Many compounds including clinically important drugs such as anticancer agents, cardiac channel blockers, cardiac drugs, antiarrhythmic agents, human immunodeficiency virus protease inhibitors, antifungals, antimalarial agents, steroid hormones, and immunosuppressants interact with P-gp (Tiberghien and Loor, 1996; Stouch and Gudmundsson, 2002). Therefore, the possible contribution of P-gp to low or erratic absorption of orally administered drugs and drug-drug interaction has been pointed out in clinical chemotherapy (Zamora et al., 1988).

The small intestine has an intrinsic nervous system, the enteric nervous system (ENS), which is recognized as an independent integrative system with structural and functional properties similar to those of the central nervous system (Furness, 2000). ENS is composed of cholinergic, adrenergic, and nonadrenergic noncholinergic (NANC) neurons (Furness, 2000). The transmitter for cholinergic and adrenergic neurons is acetylcholine and norepinephrine, respectively. The NANC neuron is a general term for several kinds of neurons whose transmitter is neither acetylcholine nor norepinephrine (Furness, 2000). Serotonin (5-HT), vasoac-
tive intestinal peptide, substance P, somatostatin, and so on are well known as transmitters for NANC neurons (Cooke, 1994). Over 95% of the 5-HT found in the body is contained in the enterochromaffin cells of the gastrointestinal mucosa. 5-HT is also located within neurons in ENS (Gershon et al., 1965). Cloning studies have revealed the existence of at least seven 5-HT receptors subdivided into 14 subtypes, most of them being present in the gastrointestinal tract (Li et al., 2001). Furthermore, electrophysiological studies have revealed the presence of at least three excitatory subtypes of the 5-HT receptor (5-HT1A, 5-HT2, and 5-HT3) on myenteric neurons of the guinea pig intestine (Nagakura et al., 1997). The presence of multiple receptor subtypes of 5-HT makes it difficult to elucidate the role of 5-HT in the gut. However, 5-HT has been shown to cause the secretion of water and electrolytes in the gut (Melean and Copaur, 1998) and to inhibit the Na⁺-dependent transport of sugars or amino acids located in the brush-border membrane (Arruebo et al., 1989; Salvador et al., 1997). 5-HT levels in the inflamed clonic mucosa were markedly decreased in human inflammatory bowel disease such as ulcerative colitis and Crohn’s disease (Magro et al., 2002), suggesting that ENS including 5-HT may have a significant role in the process of inflammatory change. A large body of evidence has been gathered which suggests that 5-HT acts as an important neurotransmitter and hormone/paracrine agent mediating enteric functions (Kadowaki et al., 1993), although the exact physiologic role of 5-HT in the bowel is not yet fully clarified.

The effect of ENS on small intestinal functions has been intensively studied in respect to the regulation of the smooth muscle (Furness, 2000) and the transport of water and/or electrolytes (Cooke and Reddix, 1994). However, information about the effect of ENS on the membrane permeability of the gastrointestinal tract is very limited (Hayden and Carey, 2000; Neunlist et al., 2003), and the regulation of drug absorption by ENS has never been systematically evaluated. In a previous study, we have investigated the effect of ENS on the absorption of a poorly absorbable compound and showed that it was suppressed or enhanced by the stimulation of adrenergic or cholinergic neurons, respectively (Higaki et al., 2004).

In the present study, we focused on 5-HT, one of the neurotransmitters in ENS regulating the function of the small intestine, and investigated the effect of 5-HT depletion on the transport activity of P-gp by employing quinidine, a typical substrate for P-gp, as a model compound.

**Materials and Methods**

**Materials**

Quinidine sulfate dihydrate and (±)-verapamil hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). DL-p-Chlorophenylalanine (PCPA) was purchased from Nacalai Tesque (Kyoto, Japan).

**Animals**

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25°C and 55% humidity, were allowed free access to standard laboratory chow (Clea Japan, Tokyo, Japan) and water. They were fasted overnight prior to the experiment, but were allowed free access to water. Rats weighing 250 to 300 g were randomly assigned to each experimental group. Our investigations were performed after approval from the local ethical committee at Okayama University and in accordance with Principles of Laboratory Animal Care (National Institutes of Health publication 85-23).

**PCPA Treatments**

5-HT-depleted rats were prepared following a method reported by Weber (1970) with minor modification. Briefly, PCPA (400 mg/kg) suspended in 1% polysorbate 80 saline was intraperitoneally injected into rats for 4 days. All experiments were performed 24 h after the last injection of PCPA. The same volume of 1% polysorbate 80 saline was intraperitoneally injected into the control rats.

**Preparation of Rat Jejunal Sheets**

After rats were anesthetized by the intraperitoneal injection of sodium pentobarbital (50 mg/kg), the jejunal segment 30 cm below the ligament of Treitz was removed and rinsed in ice-cold saline. After the segment was opened along the mesenteric border, intestinal contents were washed out with ice-cold saline. Immediately, the muscularis propria was stripped off, and three to four jejunal sheets approximately 5 cm in length without Peyer’s patches were isolated from each rat (Emi et al., 1986).

**In Vitro Intestinal Transport Study**

An intestinal sheet prepared as described above was mounted in a diffusion chamber (Corning Coaster Japan, Tokyo, Japan) with a 1.25-cm² exposed area. Ringer’s solution, containing 1.2 mM NaH₂PO₄, 125 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 10 mM NaHCO₃, and 2 mg/ml D-glucose was gassed with 95% O₂ and 5% CO₂ for 15 min and adjusted to pH 7.4 with a few drops of 1 N NaOH and then placed into both donor and receptor compartments. During the entire experiment, Ringer’s solution was gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. It was confirmed that the pH value of Ringer’s solution was kept around 7.4 until the end of the transport study. Transmucosal potential difference (PD) and short-circuit current were measured by a short-circuit current amplifier (CEZ-9000; Nihon Kohden Co., Tokyo, Japan) at 10-min intervals. The tissue electrical resistance (Rm) was calculated following Ohm’s law. After preincubation for 25 min to stabilize the electrical condition of tissues, the solution in the donor side was exchanged with the drug solution (quinidine, 500 μM). Once the transport experiments started, the solutions in both sides were circulated by gas lift with 95% O₂ and 5% CO₂ throughout the transport studies. Samples of 1 ml were drawn out of the receptor side at 10-min intervals for 90 min. An equal volume of Ringer’s solution was immediately added to the receptor side after each sampling. The drug concentration in the receptor side was determined by HPLC.

In the case of inhibition studies, verapamil (500 μM), an inhibitor for P-gp, was added with quinidine (50 μM) to the donor side (serosal side). Because a high concentration of verapamil (over 1 mM) changes the membrane permeability (Emi et al., 1988), we employed 500 μM verapamil and 50 μM quinidine, which could also allow us to assess the contribution of carrier-mediated transport more clearly.

To estimate the contribution of transcellular or paracellular transport to the secretion of quinidine (500 μM), the transport studies were performed under voltage-clamped conditions, where PD was clamped to arbitrary values (~10 to 20 mV). According to Schultz and Zalusky (1964), transmembrane secretory flux (Jₘαₜ) is represented by the following equation:

![Equation](1)

where Jₘαₜ means the transcellular flux that is independent of PD across the membrane (Vₘ), and Jₚₜ is the paracellular flux of ionized molecules dependent upon the value of Vₘ. ρₜ indicates the Jₚₜ under the short-circuited condition and ς is expressed as follows:

![Equation](2)
where $z$, $F$, $R$, and $T$ indicate ionic valency, the Faraday constant, the gas constant, and absolute temperature, respectively. 

**Preparation of Brush-Border Membrane Vesicles (BBMV)**

BBMV were obtained using the method reported by Kessler et al. (1978) with minor modification. Jejunal mucosa (about 2 g) scraped off by a glass rod was homogenized in a Waring blender for 3 min in 19 ml/g tissue of buffer A (pH 7.1) containing 50 mM mannitol and 2 mM Tris-HCl. Then, 200 μl/g tissue of 1 M CaCl$_2$ was added to the resulting mixture, which was subsequently incubated on ice for 15 min and centrifuged at 27,000 g for 30 min, the pellet was resuspended in 20 ml of buffer B (pH 7.5) containing 100 mM mannitol and 10 mM HEPES-Tris, and the suspension was homogenized with a glass Teflon homogenizer for 10 strokes. The homogenate was centrifuged at 27,000g for 30 min. The final pellets were resuspended in 1 ml of buffer C (pH 6.5) containing 100 mM mannitol and 10 mM MES-Tris, and the resulting suspensions were passed through a 27-gauge needle. The BBMV were stored at −85°C prior to use. The activity of alkaline phosphatase determined with the method of Murer et al. (1976) indicated that the final BBMV were purified around 15-fold compared with the corresponding mucosal homogenate in both control and 5-HT-depleted rats [control rats, BBMV 626.6 ± 73.2, homogenate 412.1 ± 2.1 (p < 0.01); 5-HT-depleted rats, BBMV 641.2 ± 73.8, homogenate 43.0 ± 3.4 μmol/min/mg protein (p < 0.01)].

**Western Blot Analysis**

All the equipment and chemicals used in the Western blot analysis were obtained from Bio-Rad (Hercules, CA) unless otherwise specified. BBMV resuspended in the sample buffer were separated by SDS-polyacrylamide gel electrophoresis using 12.5% polyacrylamide gel (Ready Gel J; Bio-Rad) according to the method of Laemmli (1970) and transferred to nitrocellulose membranes. The blots were blocked with phosphate-buffered saline containing 10% nonfat milk by a 1.5-h incubation at room temperature and incubated with the P-gp monoclonal antibody C-219 (Alexis Corporation, La ¨ufelfingen, Switzerland) or the villin polyclonal antibody C-19 (Santa Cruz Bio-technology, Inc., Santa Cruz, CA) for 1 h. C-219 recognizes an epitope common to each of the P-gp isoforms. The blots were then incubated for 1 h with anti-mouse or anti-goat horseradish peroxidase antibody (Kirkegaard and Perry Laboratories, Guildford, UK). The blots were developed with an ECL kit (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and exposed to ECL hyperfilm (Amersham Biosciences UK Ltd.). The quantification of bands was performed by densiometric analysis using the Scion image (Scion Corporation, Frederick, MD).

**Analytical Procedure**

**Quinidine.** Quinidine in samples obtained in the in vitro transport study was determined by HPLC according to the method reported by Emi et al. (1998). The HPLC system consists of a model LC-6A HPLC pump (Shimadzu, Kyoto, Japan), a model SIL-9A system controller (Shimadzu), and a model RF-535 fluorescence detector (Shimadzu) set at 349 nm (excitation) and 444 nm (emission). A CAPCELLPAC C18 column (150 × 4.6 mm i.d.; pore size, 120 Å; particle size, 5 μm; Shiseido Co., Tokyo) was used at room temperature. The mobile phase, 50 mM ammonium acetate (pH 2), and acetonitrile, 50:50 (v/v) was delivered at 1.0 ml/min. The coefficient of variation for the standard curve ranged from 0.3 to 26.7%, and the squared correlation coefficient ($r^2$) was over 0.999.

**5-HT in Jejunal Segments.** 5-HT was determined by the method reported by Lakshmana and Raju (1997) with minor modification. Jejunal sheets prepared as described before were minced and homogenized in a 7-fold volume of 7.14% ascorbic acid containing isoproterenol as an internal standard. Again, the resulting mixture was homogenized with the equivalent volume of 30% HClO$_4$. All the processes described above were performed on ice. After centrifugation at 9000g for 15 min, the supernatant was filtered through a millipore filter (0.22 μm, Millex-HV, Millipore Corporation, Billerica, MA), and 20 μl of filtrate was injected onto the HPLC system described above where a fluorescence detector was set at wavelengths of 280 and 315 nm for excitation and emission, respectively. An Inertsil ODS-3 column (150 × 4.6 mm i.d.; pore size, 120 Å; particle size, 5 μm; GL Sciences, Inc. Tokyo, Japan) was used for 5-HT at room temperature, and the mobile phase, 20 mM sodium acetate (pH 3.9), methanol, and heptane sulfonic acid, 800:200:0.1 (v/v) was delivered at 1.0 ml/min. The coefficient of variation for the standard curve of 5-HT ranged from 0.5 to 10.5%, and $r^2$ was over 0.999.

**ATP and Protein in Jejunal Mucosa.** ATP was assayed following the method described by Yang et al. (1999). Briefly, jejunal mucosa was vortex-mixed with ice-cold 1 M HClO$_4$ containing 1 mM EDTA, and the resulting mixture was centrifuged for 10 min at 10,000g at 0°C. The resulting pellet was used for the protein assay by the method of Lowry et al. (1951). The supernatant was neutralized with ice-cold 2.2 M KHCO$_3$ and centrifuged for 10 min at 10,000g at 0°C to remove precipitate of KCIO$_4$. The supernatant was stored at −85°C until the analysis. Bioluminescence was measured with a Lumicounter ATP-237 (Toyo Kagaku Industry, Tokyo, Japan) based on the luciferin/luciferase reaction, using an ATP detection kit (Molecular Probes, Eugene, OR).

**Data Analysis**

The cumulative amount transported to the receptor side was calculated according to the equation:

$$Q(t_n) = \sum_{n=1}^{i} C(t_{n-1}) + 6C(t_n)$$  \hspace{1cm} (3)

where $Q(t_n)$ and $C(t_n)$ indicate the cumulative amount transported to the receptor side and the concentration in the receptor side at time $t_n$, respectively. Transmural unidirectional flux rate ($J_m$, from the serosal to mucosal side; $J_m$, from the mucosal to serosal side) was calculated according to the equation:

$$J_m \text{ or } J_m = \frac{\Delta Q}{\Delta t \times A}$$  \hspace{1cm} (4)

where $Q$, $\Delta Q/\Delta t$, and $A$ are the cumulative amount of drug transferred at time $t$, the slope of the linear portion of the $Q$-time graph, and the surface area of the intestinal sheet, respectively.

**Statistical Analysis**

Results are expressed as the mean ± S.E. of three or more experiments. Analysis of variance was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was determined with Dunnett’s method, Student’s $t$ test, or a paired Student’s $t$ test.

**Results**

**Effect of 5-HT Depletion on Quinidine Transport across Rat Jejunum.** To investigate the effect of 5-HT depletion on the transmucosal transport of quinidine, 5-HT-depleted rats were prepared by intraperitoneal administration of PCPA (400 mg/kg for 4 days). A remarkable decrease in 5-HT in jejunum (0.15 ± 0.01 μg/g tissue, $p < 0.01$), just 5.10 ± 0.13% of the control value (2.94 ± 0.34 μg/g tissue), was confirmed. Therefore, PCPA-treated rats were used for all the experiments as 5-HT-depleted rats. The transport activity of P-gp was evaluated based on the secretory transport of quinidine, a typical substrate for P-gp. The unidirectional transport of quinidine (500 μM) was estimated by an in vitro transport study (Fig. 1). In control rats, the secretion of
quinidine was predominant ($J_{ms}$, 45.2 ± 15.2 pmol/min/cm²; $J_{sm}$, 173.1 ± 33.1 pmol/min/cm², p < 0.01), confirming that quinidine was secreted into the mucosal side in rat jejunum by P-gp. 5-HT depletion significantly increased both the absorptive and secretory transport of quinidine ($J_{ms}$, 235.3 ± 23.3 pmol/min/cm², p < 0.01 compared with control; $J_{sm}$, 466.9 ± 61.8 pmol/min/cm², p < 0.01 compared with control and $J_{ms}$ of 5-HT-depleted rats) (Fig. 1). Enhancement of quinidine transport, which was direction-independent, suggests that passive diffusion of quinidine could be increased by depletion of 5-HT in jejunum. On the other hand, the secretory transport of quinidine was still predominant and the increase in the rate of transport was also larger for $J_{ms}$ (293.8 ± 70.1 pmol/min/cm²) than for $J_{sm}$ (190.1 ± 27.8 pmol/min/cm²) indicating that the secretion of quinidine could be selectively enhanced under 5-HT-depleted conditions.

**Effect of 5-HT Depletion on Rm across Rat Jejunum.** As it was suggested that the transport of quinidine via passive diffusion would be enhanced in 5-HT-depleted rats, the effect of 5-HT depletion on Rm across the rat jejunum, which is a parameter reflecting passive diffusion via the paracellular route, was investigated (Fig. 2). Throughout the experiments, Rm of jejunum was significantly much smaller for 5-HT-depleted rats than control rats, indicating that the enhanced permeation of quinidine observed in Fig. 1 could be partly attributed to the increase in transport via passive diffusion through the paracellular route.

**Permeation Routes Responsible for the Enhanced Secretory Transport.** To identify the routes by which the secretory transport of quinidine was enhanced, we estimated the secretory transport of quinidine under voltage-clamped conditions (Fig. 3). In control and 5-HT-depleted rats, $J_{sm}$ of quinidine decreased with the increase in the value of $V_t$, externally applied potential, and a significant correlation was obtained between $J_{sm}$ and the value of $\xi$ (control rats, $J_{sm} = 84.5\xi + 36.2$, $r = 0.998$, $p < 0.05$; 5-HT-depleted rats, $J_{sm} = 221.2\xi + 226.9$, $r = 0.962$, $p < 0.05$). As shown in Fig. 3, 5-HT depletion increased both the slope and y-intercept of the regression line, clearly indicating the enhanced transport of quinidine via the paracellular route and the transcellular route, respectively.

**Transport Activity of P-gp under 5-HT-Depleted Conditions.** The transport activity of P-gp was determined by estimating the verapamil-sensitive secretory transport of quinidine (Table 1). Two jejunal sheets were prepared from a single rat. One was utilized for the control study and the other for the inhibition study using verapamil, an inhibitor for P-gp. Although large variability was found in $J_{sm}$ for control rats, the secretory transport of quinidine was signif-

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**Fig. 1.** Effect of 5-HT depletion on quinidine transport across rat jejunum. Absorptive (mucosal to serosal, M to S) or secretory (serosal to mucosal, M to S) transport of quinidine (500 μM) was investigated in an in vitro intestinal transport study. Results are expressed as the mean with the bar showing the S.E. for four experiments. *, $p < 0.05$; ‡, $p < 0.01$ compared with the absorptive transport in each group of rats. †, $p < 0.05$; ‡, $p < 0.01$ compared with control rats in each direction of transport. Control rats: ○, M to S; ●, S to M. 5-HT-depleted rats: □, M to S; ■, S to M.

**Fig. 2.** Effect of 5-HT depletion on Rm in rat jejunum. PD and short-circuit current values were measured at 10-min intervals. Rm was calculated following Ohm’s law. Results are expressed as the mean with the bar showing the S.E. for four experiments. *, $p < 0.05$; ‡, $p < 0.01$ compared with control rats. ○, control rats; ●, 5-HT-depleted rats.

**Fig. 3.** Secretory transport of quinidine in rat jejunum under voltage-clamped conditions. Transport studies were performed with PD clamped from −10 to 20 mV. The concentration of quinidine was 500 μM. $J_{sm}$ was calculated following the method described under Materials and Methods. Solid lines were obtained by least-squares regression. Results are expressed as the mean with the bar showing the S.E. for three to five experiments. *, $p < 0.01$ compared with control rats. ○, control rats; ●, 5-HT-depleted rats.
TABLE 1
Enhancement of verapamil-sensitive secretion of quinidine by 5-HT depletion in rat jejunum

<table>
<thead>
<tr>
<th></th>
<th>Control Rat</th>
<th>5-HT-Depleted Rat</th>
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<tbody>
<tr>
<td>J_{sm} pmol/min/cm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>49.6 ± 17.6</td>
<td>93.1 ± 12.5</td>
</tr>
<tr>
<td>+ Verapamil</td>
<td>29.0 ± 14.9</td>
<td>51.2 ± 8.9</td>
</tr>
<tr>
<td>Verapamil-sensitive transport</td>
<td>20.6 ± 3.4</td>
<td>41.9 ± 4.8</td>
</tr>
</tbody>
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*p < 0.05, compared with control rat.
*p < 0.05, compared with the corresponding control study.

Effect of 5-HT Addition on Quinidine Transport across Rat Jejunum. As it was shown that 5-HT depletion increased the secretory transport of quinidine, we investigated the effect of the addition of 5-HT on the transport of quinidine in 5-HT-depleted and control rats (Fig. 4A). No significant change was observed in quinidine transport with 5-HT in the mucosal or serosal side, although the addition of 5-HT tended to increase quinidine transport in both directions in 5-HT-depleted rats. No significant change was observed in Rm, except that addition of 5-HT tended to decrease Rm in control rats (Fig. 4B).

Effect of 5-HT Depletion on ATP Amount in Rat Jejunal Mucosa. As it was recognized that the transport activity of P-gp was enhanced under 5-HT-depleted conditions, the level of ATP in rat jejunal mucosa was determined to estimate the ATP level, but no significant difference was observed between control rats and 5-HT-depleted rats. As shown in Fig. 5C, 5-HT depletion did not change the intracellular ATP level either.

P-gp Expression in Jejunal Brush-Border Membrane. To estimate the P-gp expression level in the brush-border membrane of jejunal epithelial cells, Western blot analysis was performed for both control rats and 5-HT-depleted rats using villin as an internal standard (Fig. 6). Figure 6A clearly shows that P-gp expression was significantly induced in 5-HT-depleted rats compared with a control group. On the other hand, the expression level of villin was not changed by 5-HT depletion, as shown in Fig. 6B. Villin is a major structural component of the brush-border absorptive cells. Therefore, it was chosen as a control protein to assess the relative expression of P-gp in the membrane under the different conditions. Samples were applied in four different protein amounts to evaluate P-gp expression quantitatively, and the densities of bands estimated using Scion image densitometry were significantly correlated with the applied amounts of protein (P-gp, r > 0.916, p < 0.002; villin, r > 0.969, p < 0.001). Using the ratio of P-gp to villin in density, the expression level of P-gp was calculated as a percentage of that of control rats. The expression level of P-gp in the brush-border membrane of 5-HT-depleted rats increased up to around two times the control value (Fig. 7). This result evidently shows that the enhanced activity of P-gp in 5-HT-depleted rats is at least partly ascribed to the induction of P-gp expression in the brush-border membrane.

Discussion

P-gp is widely expressed in normal tissues including the small intestine (Kondratov et al., 2001; Stouch and Gudmundsson, 2002), but as P-gp effluxes its substrates from epithelial cells into the intestinal lumen, their arrival in epithelial mucosa and total amount of protein in rat jejunum, respectively. These parameters would be critical for normalizing the level of ATP, but no significant difference was observed between control rats and 5-HT-depleted rats. As shown in Fig. 5C, 5-HT depletion did not change the intracellular ATP level either.
blood would be decreased. Therefore, understanding and regulating P-gp function may lead to improvements in clinical chemotherapy. Gastrointestinal function is regulated by ENS which consists of the myenteric plexus and submucosal plexus, and serotoninergic neurons are only present on the plexus lying closest to the circular muscles among the submucosal plexus (Hayden and Carey, 2000). As there is approximately 10 times more 5-HT in the small intestine than the brain (Weber, 1970), 5-HT is considered to play the major role in gastrointestinal tract. 5-HT causes net water and electrolyte secretion in the small intestine (Mclean and Coupar, 1998). Recent studies also indicated that 5-HT inhibited the Na+/H+-dependent transport of sugars or amino acids (Arruebo et al., 1989; Salvador et al., 1997). However, there is little information about the effect of 5-HT on drug absorption. In the present study, we tried to estimate the role of 5-HT in P-gp-mediated drug transport using 5-HT-depleted rats.

We prepared 5-HT-depleted rats by treatment with PCPA, which depletes 5-HT via a specific inhibition of tryptophan hydroxylase in 5-HT biosynthesis. As this inhibitory effect is specific for 5-HT, other catecholamines such as norepinephrine and dopamine are not affected by PCPA (Koe and Weissman, 1966). As the amount of 5-HT in the intestinal mucosa was significantly reduced to approximately 5% of the control level, we were able to investigate the effect of chronic 5-HT depletion on the function of P-gp.

The transport of quinidine, which is both an inhibitor and substrate of P-gp (Akiyama et al., 1987; Emi et al., 1998), was examined using jejunal sheets prepared from 5-HT-depleted rats. Although the transport of quinidine in 5-HT-depleted rats was secretory-predominant, the permeability in both the secretory and absorptive directions was significantly increased (Fig. 1), suggesting that quinidine transport via passive diffusion was enhanced in common. The enhancement of verapamil-insensitive transport shown in Table 1 can be explained by enhanced passive diffusion. The decrease in Rm down to about 60% of the control value indicated that the increased permeability of quinidine via passive diffusion was partly attributable to an expanded paracellular route (Fig. 2). The membrane permeation of drugs via passive diffusion is through transcellular and paracellular routes (Frizzell and Schultz, 1972). In the latter route, the tight junctions between epithelial cells play an important role in the paracellular diffusion, so structural changes to tight junctions affect membrane permeation via the paracellular route (Ward et al., 2000). Generally, it has been postulated that increased intracellular Ca^{2+} concentrations lead to the widening of tight junctions via a series of reactions and structural changes triggered by the activation of calmodulin-dependent kinase (Ward et al., 2000). 5-HT is known to increase the intracellular Ca^{2+} level due to uptake from the extracellular compartment or release from the intracellular store via the

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**Fig. 5.** Effect of 5-HT depletion on ATP level in rat jejunal mucosa. Wet weight of jejunal mucosa (A), total protein in jejunal mucosa (B), and ATP level in jejunal mucosa (C) were expressed as the mean with the bar showing the S.E. for 8 to 10 experiments. □, control rats; ■, 5-HT-depleted rats.

**Fig. 6.** Immunodetection of P-gp and villin in rat jejunal brush-border membrane. SDS-polyacrylamide gel electrophoresis was performed with four different amounts of protein (3.75, 7.5, 15, and 30 μg of total protein). C and D represent mean control rats and 5-HT-depleted rats, respectively. Immunodetection of P-gp (A) and villin (B) was performed with monoclonal antibody C-219 and C-19, respectively.

**Fig. 7.** Quantitative estimation of P-gp expression in rat jejunal brush-border membrane. Immunoreactive bands detected by Western blot analysis in Fig. 6 were evaluated with Scion image. P-gp levels are expressed as the ratio of P-gp to villin in density of bands. Results are expressed as the mean with the bar showing the S.E. for four experiments for each trial. *, p < 0.05 compared with control rats. □, control rats; ■, 5-HT-depleted rats.
activation of phospholipase C (Zinner et al., 1986; Lee and Wu, 1999). Therefore, the intracellular Ca\(^{2+}\) concentration was expected to be lower under 5-HT-depleted conditions, but the results obtained (Fig. 2) suggested an increase in intracellular Ca\(^{2+}\). So, in future studies, the effect of 5-HT depletion on the intracellular Ca\(^{2+}\) concentration has to be investigated to clarify the mechanisms of enhanced passive diffusion via the paracellular pathway.

Even though passive diffusion was enhanced, the transport of quinidine was still predominant in the secretory direction (Fig. 1), indicating that 5-HT depletion could also selectively enhance the secretory transport of quinidine. Then, the enhanced secretory transport of quinidine was estimated by the voltage-clamp method, which allows one to evaluate the transport via each pathway (Schultz and Zalusky, 1964). The result has shown that 5-HT depletion enhanced quinidine transport via the transcellular route as well as paracellular route (Fig. 3). The enhanced secretory transport of quinidine via the transcellular route might be partially attributed to the enhanced membrane permeability probably due to an increase in membrane fluidity. However, the inhibition study using verapamil clearly showed that the secretion of quinidine via P-gp was significantly enhanced under 5-HT-depleted conditions (Table 1). Quantitative Western blot analysis showed that the expression level of P-gp in the jejunal brush-border membrane of 5-HT-depleted rats increased 2-fold compared with that of control rats (Fig. 7). As the secretion of quinidine via P-gp was also enhanced around two times compared with the control (Table 1), the enhanced transport via P-gp could be explained by the induced expression of P-gp in the jejunal brush-border membrane in 5-HT-depleted rats. Although the involvement of other transporters such as organic cation transporter 1 cannot be excluded, further studies are needed to clarify the contribution of other transporters.

The details of the mechanisms for the enhancement of P-gp expression remain to be clarified, but an increased mRNA level has to be considered under 5-HT-depleted conditions. It might also be attributed to the increased recruitment of P-gp from pre-existing intracellular pools as reported following stimulation with cAMP in the liver (Kipp et al., 2001). 5-HT is considered to increase the intracellular cAMP level via 5-HT receptors, but the possibility that chronic 5-HT depletion enhances trafficking cannot be excluded, as is the case for the intracellular Ca\(^{2+}\) level. Moreover, phosphorylation and glycosylation of P-gp and membrane conditions where P-gp is located are suggested to be factors regulating the activity and expression of P-gp (Castro et al., 1999; Sharam et al., 1999; Gribar et al., 2000). Therefore, further investigation is needed to clarify the mechanisms behind the induction of P-gp expression in the brush-border membrane by 5-HT depletion.

Besides the increase in the expression level, other possible mechanisms might be considered. Many reports suggested that protein kinase C (PKC) could be involved in the regulation of P-gp function (Ma et al., 1991; Castro et al., 1999), although recent reports showed that phosphorylation of MDR1 did not play an essential role in its drug-transporting activity (Szabo et al., 1997). The important point to be made is that 5-HT is deeply involved in the regulation of PKC activity. It was suggested that PKC was activated through a series of reactions including an increase in cAMP and/or diacylglycerol after the binding of 5-HT to several subtypes of 5-HT receptors (Nagakura et al., 1997; Furness, 2000). Chronic depletion of 5-HT might cause some effects that would be different from those expected with the transient depletion or inhibition of 5-HT, but several mechanisms where 5-HT is involved must be perturbed.

Chronic depletion of 5-HT might result in a number of biological changes that are unexpected, but we confirmed that the wet weight of intestinal mucosa (Fig. 5A) and the total amount of protein contained in the intestinal mucosa (Fig. 5B) were not significantly affected. These parameters are very important when estimating the expression level of P-gp and intracellular ATP level, because the values determined for them must be normalized using parameters such as the total protein amount. The intracellular ATP level was not affected by 5-HT depletion (Fig. 5C), indicating that the ATP level has nothing to do with the enhanced P-gp activity under 5-HT-depleted conditions.

The addition of 5-HT to the jejunal sheets isolated from 5-HT-depleted rats did not have any significant effect (Fig. 4). Jejunal tissues employed in the present study have serotonergic neurons among the submucosal plexus, even though the myenteric plexus was removed by stripping the external muscle layers (Cooke and Reddix, 1994; Hayden and Carey, 2000). Therefore, the addition of 5-HT was expected to exert some effect via serotoninergic neurons. However, no significant changes were observed (Fig. 4), suggesting that the transient addition might not be enough to reverse the biological change caused by chronic depletion of 5-HT. Chronic depletion of 5-HT would cause some subsequent changes, which might be directly connected with the induction of P-gp expression. As the expression of P-gp in the liver and kidney was not induced under the same conditions where P-gp in the small intestine was induced (data not shown), the induction of P-gp would be specific to the small intestine. This result suggests that chronic depletion of 5-HT could be a trigger for the induction of P-gp expression in the small intestine where 5-HT plays an important role. There is a need to clarify the mechanisms by which chronic depletion of 5-HT leads to the induction of P-gp expression.

In conclusion, chronic 5-HT depletion significantly enhanced the secretory transport of quinidine, which could be attributed to passive diffusion and to active transport via P-gp. The induction of P-gp expression in the brush-border membrane could be responsible for the increase in P-gp activity, although the mechanisms behind the induction remain to be clarified.

References


