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Transport Mechanism of Intestinal Absorption of μ Opioid Receptor Agonists and Contribution of P-Glycoprotein in Rats and Human Intestinal Epithelial Caco-2

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Abstract

1.1. Introduction

The μ opioid receptor agonists, morphine and loperamide, are widely used orally and are suggested to be P-glycoprotein (P-gp) substrates. P-gp is expressed in the brain, intestine, and various tissues in human and rats. In the intestine, P-gp limits the absorption of certain drugs such as opioids; however, the underlying mechanism has not been elucidated. The aim of the present study was to examine the intestinal transport characteristics of morphine and loperamide and the role of P-gp in their transport process.

1.2. Method

Transcellular transport studies were conducted using isolated rat intestinal tissue mounted in an Ussing-type chamber. Bidirectional permeability and inhibition transport studies were performed using Caco-2 cell lines. The intestinal absorption was examined by an *in situ* closed-loop method in rats.

1.3. Results

Loperamide showed secretory transport across rat intestinal tissue and Caco-2 cells, and P-gp substrates cyclosporine A and rhodamine 123 inhibited this transport. In the intestinal loop experiment in rats, the accumulation of loperamide in the intestinal tissue increased upon adding cyclosporine A and rhodamine 123. In contrast, morphine showed no directional transport and P-gp inhibitory effects across rat intestinal tissue. In Caco-2 cells, morphine transport was found to be secretory-directed and this transport was inhibited by cyclosporine A and rhodamine 123, but to a much lesser extent than that of loperamide. Morphine disappearance and accumulation were unaffected upon the addition of cyclosporine A and rhodamine 123.

1.4. Conclusion

These results suggest that P-gp contributes significantly to the secretory transport of loperamide but negligibly to that of morphine in the small intestine. In conclusion, intestinal transport of both morphine and loperamide is found to be secretory-directed. P-gp partially contributes to this secretory-directed transport. Thus, P-gp is prominent in loperamide rather than morphine transport.

Introduction

Morphine and loperamide are μ opioid receptor agonists with a long history of clinical use worldwide [1]. Morphine is an analgesic drug widely used to alleviate cancer-related pain. It can be taken orally, is absorbed in the intestine, and permeates into the brain, where it binds μ opioid receptors to exert its central pharmacological effect. Further, morphine peripherally binds to the intestinal μ opioid receptors, causing constipation (an adverse effect of morphine) [2]. Loperamide is an antidiarrheal agent that exerts its pharmacological activity by agonistically binding to μ opioid receptors in the intestine, similar to the mechanism of morphine's adverse effect. However, it has little or no central pharmacological effect because it does not readily permeate into the brain [3,4]. The difference in the pharmacological

effects of morphine and loperamide can be attributed to the differences in their tissue distributions. The degree of interaction of each drug with P-glycoprotein (P-gp) at the blood-brain barrier (BBB) may explain this difference [5]. P-gp is a transmembrane adenosine triphosphate (ATP)-driven efflux pump encoded by multidrug resistance gene (*MDR1* or *ABCB1*). It is mainly expressed in the transporting epithelia of various human tissues, including the intestine, liver, kidney, and BBB, where it actively transports its substrates out of the cell [6-8]. P-gp substrates

include major antiarrhythmics (verapamil and bepridil); anticoagulants (apixaban, dabigatran, and propranolol); and antihypertensive drugs (aliskiren, diltiazem, losartan, and talinolol); immunosuppressive calcineurin inhibitors cyclosporine and tacrolimus [9]; and narcotic analgesic morphine [10]; however, the contribution of P-gp to the total amount of intestinal absorption varies.

Loperamide is a high-affinity P-gp substrate. It is effectively pumped out by P-gp at the BBB; thus, pharmacologically effective concentrations are not achieved in the brain [3]. Morphine is also suggested to be a substrate of P-gp, because it has been shown to produce greater analgesia in P-gp-knockout mice than in wild-type mice [11]; however, morphine disposition is not considered critically dependent on P-gp [3]. P-gp is expressed in the small intestine as well as the BBB. In the small intestine, P-gp limits the absorption of certain drugs. The pharmacokinetics of digoxin is affected by the function of P-gp in the intestine [12]. Intestinal P-gp limits the oral bioavailability and active epithelial excretion of paclitaxel, as demonstrated by using mdr1a-knockout mice [13]. In rats, several β-blockers are actively secreted from the small intestinal epithelium into the lumen by P-gp, which functions as an absorption barrier, resulting in decreased blood concentrations of these drugs [14]. In addition, the absorptive transport of the serotonin antagonist azasetron is regulated by P-gp *in vitro* [15]. Intestinal transport of pitavastatin is mediated by efflux transporters such as not only P-gp but also another transporter, organic anion transporting polypeptide, as [16]. We demonstrated that the intestinal transport of quinolone antimicrobials is regulated by P-gp [17,18] in rat intestinal tissue and human intestinal cell line Caco-2. Considering its important role in intestinal absorption, P-gp could influence the absorption of opioids in the small intestine. However, the mechanisms of the intestinal absorption of such drugs and the role of intestinal P-gp in their absorption have not yet been directly demonstrated. In this study, we aimed to examine the intestinal transport characteristics of morphine and loperamide by using Caco-2 cells and rat small intestinal tissues and the role of P-gp in their transport by performing inhibition studies with rhodamine 123 and cyclosporine A (CsA).

Materials and Method

Chemicals

Loperamide hydrochloride and rhodamine 123 were purchased from Sigma Co (St. Louis, MO). Morphine was purchased from Takeda Pharmaceutical Co. Ltd. (Tokyo, Japan).CsA (Sandimmun® for i.v. infusion, 250 mg) was purchased from Novartis Pharma K.K. (Tokyo, Japan). All other reagents were commercial products of reagent grade and were used without further purification.

Animals

Wistar / ST male rats were used at the age of 6 to 8 weeks. The animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Doshisha Women's College of Liberal Arts.

Transport experiments by the Ussing-type chamber method

Rat intestinal tissue sheets were prepared as described previously [17,18]. The tissue sheets, consisting of the mucosa and most of the muscularis mucosa, were mounted vertically in an Ussing-type chamber that provided an exposed area of 0.5 cm². The volume of bathing solution on each side was 5 mL, and the temperature was maintained at 37°C. The test solution was composed of 128 mM NaCl, 5.1 mM KCl, 1.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 21 mM $NaHCO_3$, 1.3 mM KH_2PO4, 10 mM NaH_2PO_4, and 5 mM glucose at pH 7.4 or 6.0 and was gassed with

O_2 before and during the transport experiments. In the inhibition studies, modulators were added to the same side as the substrate.

Intestinal absorption study by the closed loop method

Intestinal absorption study by the closed loop method was performed as previously reported [17,18]. After the rats were anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg), their intestines were exposed by midline abdominal incisions. Closed ileal loops of 10 cm or 20 cm were prepared by ligating both ends after clearing the gut with slowly passed warmed isotonic 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (5 mM KCl, 100 mM NaCl, 10 mM MES, 85 mM mannitol, 0.01% polyethylene glycol; pH 6.4; 290 mOsm/kg osmolarity) until the effluent became clear and expelling the remaining solution by means of air pumped through a syringe. Isotonic MES buffer (50 µL/cm), including drug with or without inhibitor, was administered into the loops as a bolus. The animals were kept on a warm plate at 37°C. After 15 min or 30 min, the solution in the loops was collected and the loops were rinsed with isotonic MES buffer to give a total volume of 5 mL. After collecting the solution in the loops, the mucosa was also collected.

Transport experiments with Caco-2 cells

Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin on a Transwell microporous polycarbonate membrane (Costar, Bedford, MA) for 21-23 days and used in the transport study as described previously [19-21]. The confluent cells were washed with Hanks' balanced salt solution (HBSS) (0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM HEPES; pH 7.4 or 6.0; 315 mOsm/kg osmolarity) at 37°C, and 0.5 and 1.5 mL HBSS were added on the apical and basolateral sides of cell inserts, respectively. To measure the apical-to-basolateral (absorptive) or basolateral-to-apical (secretory) flux, a test compound was applied on the apical or basolateral side, respectively. At the designated time, 0.5 mL of the basolateral or 0.2 mL of the apical side solution was withdrawn and replaced with an equal volume of HBSS.

Analytical methods

Samples of experimental solution from the Ussing-type chamber and closed loop experiments were properly diluted in 0.1 M phosphate buffered saline (PBS). Samples of intestinal mucosa from the closed loop experiments were added to 2 mL of 0.1 M PBS and homogenized. The homogenate was centrifuged to yield the supernatant. The supernatant from the loperamide experiments was then subjected to high-performance liquid chromatography (HPLC) determination. In the case of morphine, the extraction was performed by adding 1 mL of Kolthoff buffer (50 mM $Na_2B_4O_7$ and 100 mM KH_2PO_4, pH 7.4) and 12.5 mL chloroform / isobutanol (9:1) to 1 mL of the supernatant and vortexing thoroughly. The mixture was centrifuged. The resultant water phase was removed by aspiration, and 12 mL of the chloroform / isobutanol phase was transferred to a new tube containing 2.5 mL of HCl. The tube was vortexed and centrifuged and 2.4 mL of the water phase was transferred to a new tube. The pH was adjusted to 8.7 ± 0.2 with 1 M K_2CO_3, and 2.5 mL of ethylacetate / isopropanol (9:1) was added. After centrifugation, 2.3 mL of ethylacetate / isopropanol was transferred to a new tube and dried by nitrogen gas aeration at 50°C for about 1 h. The dried pellet was reconstituted with 0.5 mL of the mobile phase for HPLC determination. The HPLC system consisted of a constant-flow pump (LC-6A; Shimadzu Co., Kyoto, Japan), a UV

detector (SPD-6A; Shimadzu Co.), an integrator (Chromatopac CR3A; Shimadzu Co.), and an automatic sample injector (SIL-6B; Shimadzu Co.); a Cosmocil 5C18 AR-II column (250 mm height × 4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan) was used as the analytical column. The conditions for morphine and loperamide analyses were according to Matsubayashi et al. [22] and Chen et al. [23] with some modifications, respectively. For morphine, the mobile phase comprised 3 mM potassium phosphate buffer (pH 5.8)-acetonitrile (95:5 [vol / vol]) solution with a column temperature of 40°C, flow rate of 1.2 mL/min, and UV detection at 210 nm. For loperamide, the mobile phase was composed of 20 mM sodium phosphate buffer (pH 2.3)-acetonitril-dimethylamine (45:55:0.08 [vol / vol / vol]) solution with a column temperature of 40°C, flow rate of 1.0 mL/min, and UV detection at 195 nm. The amount of rhodamine 123 was measured by a fluorescence detector set at 486 nm and 545 nm as the excitation and emission wavelengths, respectively.

Data analysis

Transport was estimated in terms of permeation ($\mu L/cm^2$) by dividing the amount transported ($\mu mol/cm^2$) with the initial concentration of the test compound on the donor side ($\mu mol/\mu L$). The permeability coefficient ($\mu L/cm^2/min$) was obtained from the slope of the linear portion of the plots of permeation against time (min). The secretory ratio was calculated from the permeability coefficient of absorptive transport divided by that of secretory transport. All data are expressed as the means ± SEM and statistical analysis was performed with Student's t-test. A difference of $P < 0.05$ between means was considered significant.

Results

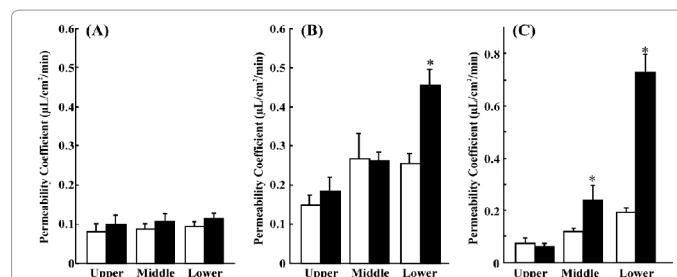#### Transport of morphine and loperamide across rat small intestinal tissues

We studied morphine and loperamide permeation in rat intestinal tissue mounted in an Ussing-type chamber. Morphine permeation did not show regional differences or directional transport (Figure 1A). Loperamide permeation showed regional dependence bi-directionally, with higher values in the ileum followed by the jejunum and duodenum, and significant secretory-directed transport in the ileum (Figure 1B). Rhodamine 123 also showed the same regional dependence as loperamide, with more prominent secretory transport (Figure 1C).

To examine P-gp involvement in opioid permeation across rat intestinal tissue, inhibition experiments were performed. Addition of CsA and rhodamine 123 did not affect morphine permeation, even at concentrations as high as 20 μM (Figure 2A). However, addition of CsA (5 μM, 20 μM) significantly decreased the secretory transport of loperamide, resulting in a significant decrease in the secretory ratio. Addition of rhodamine 123 tended to increase the absorptive transport and decrease the secretory transport of loperamide, resulting in a significant decrease in the secretory ratio (Figure 2B). Further, we examined the inhibitory effect of the opioids on rhodamine 123 transport. The secretory-directed transport of rhodamine 123 was decreased resulting in the secretory ratio by adding CsA. Addition of loperamide also significantly decreased rhodamine 123 secretion, whereas morphine had no effect on rhodamine 123 secretion (Figure 2C).

#### Absorption of morphine and loperamide from rat small intestinal loops

We examined the intestinal absorption of morphine and loperamide by an *in situ* closed loop method in rats.

Morphine disappearance and accumulation are shown in Figure 3A. It took 30 min for about 80% disappearance. No changes in disappearance and accumulation of morphine were observed upon the addition of CsA and rhodamine 123, with an exception of significant but slight increase in accumulation by CsA 20 μM.

The transport of (A) morphine (100 μM), (B) loperamide (1 mM), and (C) rhodamine 123 (20 μM) across the intestinal tissues was estimated from the time course of transport by the Ussing-type chamber method. The tissues were isolated from the upper, middle, or lower intestinal region. The experimental solution was adjusted to pH 7.4, and the temperature was maintained at 37°C. The open and closed columns represent the mucosal-to-serosal (absorptive) and serosal-to-mucosal (secretory) permeation, respectively.
Each column represents the mean ± SEM from 3-5 experiments.
*$P < 0.05$, significantly different from the corresponding mucosal-to-serosal transport by Student's t-test

Figure 1: Regional differences in morphine, loperamide, and rhodamine 123 transport across rat small intestinal tissues mounted in an Ussing-type chamber.
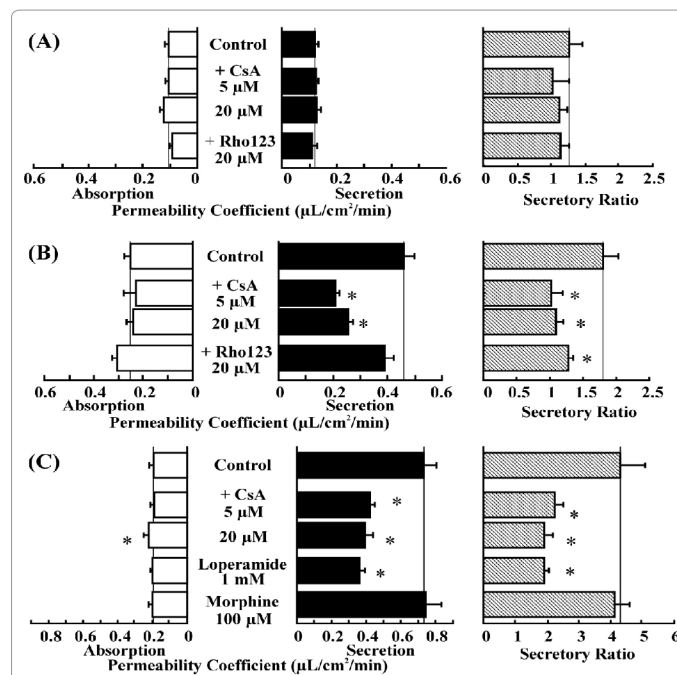
The transport of (A) morphine (100 μM), (B) loperamide (1 mM), and (C) rhodamine 123 (20 μM) across the intestine was measured in the ileal tissues with or without a P-gp substrate or inhibitor added to the donor solution. The experimental solution was adjusted to pH 7.4, and the temperature was maintained at 37°C. The open and closed columns represent mucosal-to-serosal (absorptive) and serosal-to-mucosal (secretory) permeability coefficients, respectively. The hatched columns represent the secretory ratios, calculated as the permeability coefficient of the serosal-to-mucosal direction divided by that of the mucosal-to-serosal direction.
Each column represents the mean ± SEM from 3-5 experiments.
*$P < 0.05$, significantly different from the corresponding control by Student's t-test
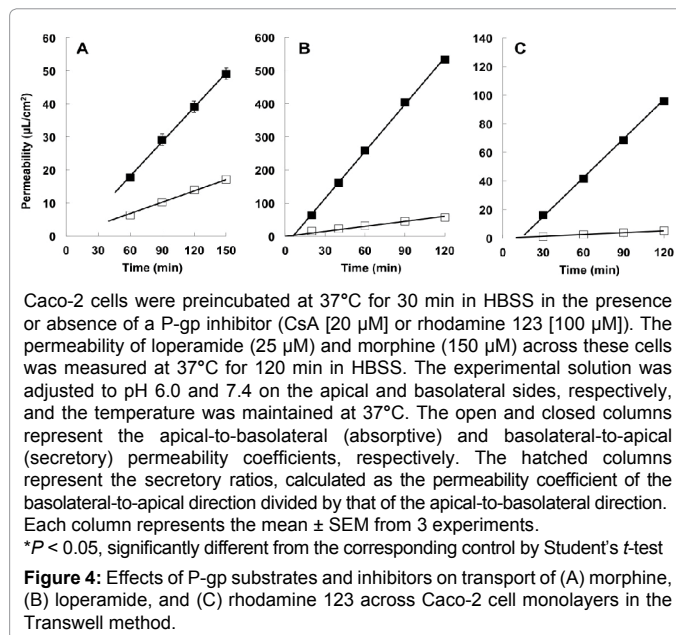
Figure 2: Effects of P-gp substrates and inhibitors on the transport of morphine, loperamide, and rhodamine 123 across isolated rat ileal tissues mounted in an Ussing-type chamber.

Loperamide disappearance and accumulation are shown in Figure 3B. It took 15 min for about 80% disappearance. Addition of CsA and rhodamine 123 did not affect the disappearance of morphine. However, the accumulation of loperamide in the intestinal tissue was significantly increased by CsA in a dose-dependent manner. Similarly, addition of rhodamine 123 significantly increased loperamide accumulation.

The inhibitory effect of morphine and loperamide on rhodamine 123 transport was evaluated in the intestinal loop experiment (Figure 3C). Addition of CsA increased rhodamine 123 disappearance and accumulation in a dose-dependent manner. Similar results were observed upon loperamide addition, while morphine addition did not affect rhodamine 123 disappearances and accumulation.

Transport of morphine and loperamide across Caco-2 cells

We studied the transepithelial transport of morphine and loperamide in Caco-2 cells (Figure 4). Results suggest that secretory transport was found to be greater than absorptive transport for morphine, loperamide, as well as rhodamine 123. The calculated secretory ratios were 1.5, 11.3, and 21.1 for morphine, loperamide, and rhodamine 123, respectively.

Caco-2 cells were preincubated at 37°C for 30 min in HBSS in the presence or absence of a P-gp inhibitor (CsA [20 μM] or rhodamine 123 [100 μM]). The permeability of loperamide (25 μM) and morphine (150 μM) across these cells was measured at 37°C for 120 min in HBSS. The experimental solution was adjusted to pH 6.0 and 7.4 on the apical and basolateral sides, respectively, and the temperature was maintained at 37°C. The open and closed columns represent the apical-to-basolateral (absorptive) and basolateral-to-apical (secretory) permeability coefficients, respectively. The hatched columns represent the secretory ratios, calculated as the permeability coefficient of the basolateral-to-apical direction divided by that of the apical-to-basolateral direction. Each column represents the mean ± SEM from 3 experiments.
*$P < 0.05$, significantly different from the corresponding control by Student's t-test

Figure 4: Effects of P-gp substrates and inhibitors on transport of (A) morphine, (B) loperamide, and (C) rhodamine 123 across Caco-2 cell monolayers in the Transwell method.

In inhibition studies, both absorptive and secretory permeations of morphine were significantly decreased by addition of CsA, resulting in no change in the secretory ratio. Addition of rhodamine 123 decreased only the secretory transport of morphine, resulting in a significant but minimal decrease in the secretory ratio (Figure 5A).
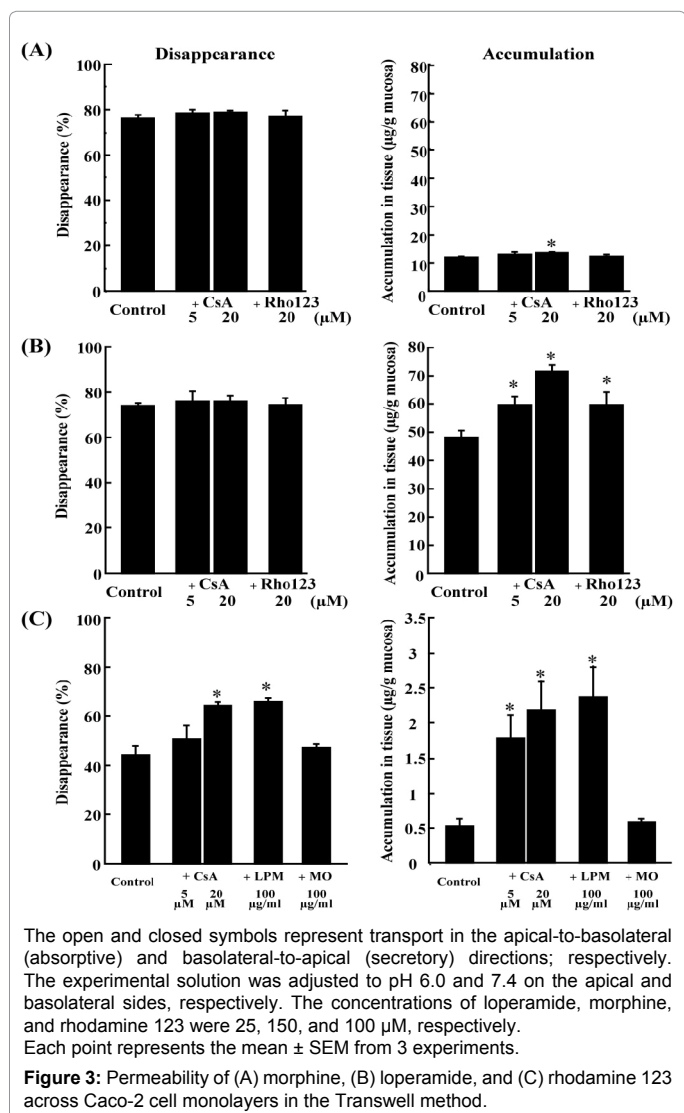
As for loperamide, addition of CsA or rhodamine 123 significantly increased the absorptive transport and decreased the secretory transport, resulting in a significant and modest decrease in the transport of rhodamine 123 (Figure 5B).
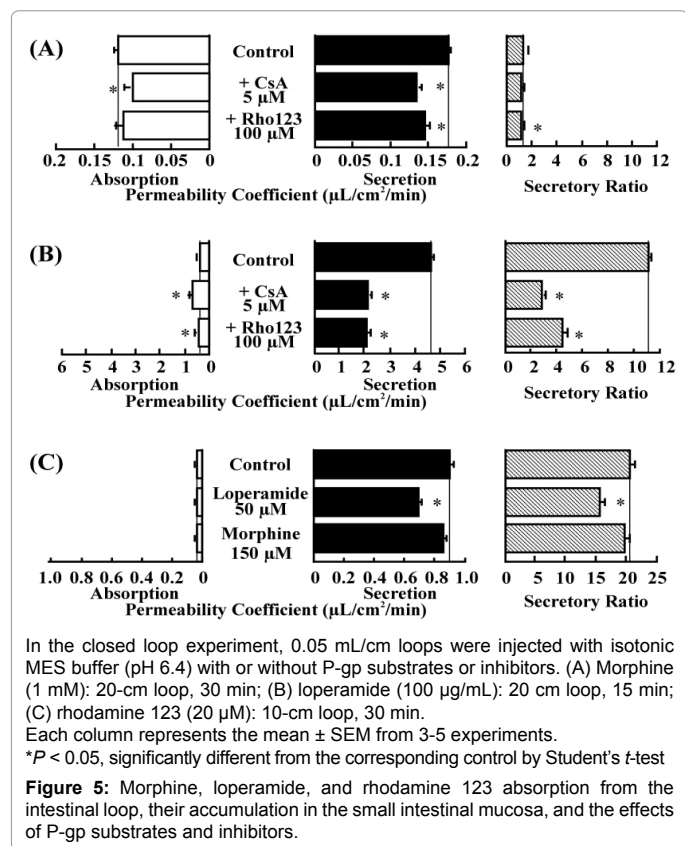
Secretory transport of rhodamine 123 was significantly decreased by loperamide, but no effects were observed in case of morphine (Figure 5C).

Discussion

Morphine and loperamide are μ opioid receptor agonists and are administered orally. Morphine, and not loperamide, has central effects in humans, likely because of differences in their penetration into the brain, with P-gp playing a key role in this process. P-gp is expressed in the intestine and is considered to act as an absorption barrier for certain drugs. However, the transport mechanisms of both opioids remain to be clarified. The aim of this study was to clarify the transport characteristics of morphine and loperamide in the small intestine and the role of P-gp in these processes.

Loperamide showed significant and rapid secretory-directed transport in rat small intestinal tissues mounted in an Ussing-type chamber, and the degree of secretion was the greatest in the lower intestinal region. This result is in good agreement with that obtained for grepafloxacin, the intestinal transport of which was found to be affected by P-gp. In addition, it is reported that the P-gp expression level in the rat small intestine is high in the lower intestinal region and low in the upper intestinal region [24,25]. The secretory-directed transport of loperamide was significantly inhibited by P-gp substrates: CsA and rhodamine 123. Therefore, it is suggested that P-gp can be responsible for the secretory transport of loperamide, which is CsA- and rhodamine 123-sensitive. Loperamide is metabolized to desmethylloperamide, and its N-methylation is catalyzed by CYP2B6, CYP2C8, and CYP2D6 in

The open and closed symbols represent transport in the apical-to-basolateral (absorptive) and basolateral-to-apical (secretory) directions; respectively. The experimental solution was adjusted to pH 6.0 and 7.4 on the apical and basolateral sides, respectively. The concentrations of loperamide, morphine, and rhodamine 123 were 25, 150, and 100 μM, respectively.
Each point represents the mean ± SEM from 3 experiments.

Figure 3: Permeability of (A) morphine, (B) loperamide, and (C) rhodamine 123 across Caco-2 cell monolayers in the Transwell method.

In the closed loop experiment, 0.05 mL/cm loops were injected with isotonic MES buffer (pH 6.4) with or without P-gp substrates or inhibitors. (A) Morphine (1 mM): 20-cm loop, 30 min; (B) loperamide (100 μg/mL): 20 cm loop, 15 min; (C) rhodamine 123 (20 μM): 10-cm loop, 30 min.
Each column represents the mean ± SEM from 3-5 experiments.
*P < 0.05, significantly different from the corresponding control by Student's t-test.

Figure 5: Morphine, loperamide, and rhodamine 123 absorption from the intestinal loop, their accumulation in the small intestinal mucosa, and the effects of P-gp substrates and inhibitors.

human liver microsomes [26]. CYP3A2 is the homolog of CYP3A4 in rats, and this enzyme is expressed not only in the liver but also in the intestine [27]. CsA is known to have dual inhibitory effects on P-gp and CYP3A [28,29]. In addition, loperamide has been reported to be metabolized by CYP3A4 in the liver [30]. Therefore, loperamide may be metabolized by CYP3A in the intestine, and the inhibitory effects of CsA on loperamide transport might be mediated mainly through CYP3A. However, CYP3A was not found to have a role in metabolism in this study owing to the following. First, we quantified the concentration of loperamide by HPLC to determine its kinetics. Second, we examined transports of both absorptive and secretory directions. Since it is thought that the interaction between the drug and the enzyme should be the same in both directions, the difference in transport and secretory ratio is attributable to the differences in transporter activity. Third, if the effect of CsA on CYP3A had been the main reason, bidirectional permeation would have increased in the Ussing-type chamber method. Lastly, the inhibitory effect was observed by using rhodamine 123 [31,32], which has a specific effect on P-gp and no effect on metabolism by CYP3A. Therefore, our results indicate that loperamide is effluxed by P-gp, in part, in the small intestine.

We also used Caco-2 cells to confirm the participation of P-gp in intestinal permeation because Caco-2 cells are derived from humans, and have minimal activity of metabolic enzymes including CYP3A4 [13,33,34]. In the Transwell method, profound secretion of loperamide was observed and this secretion was inhibited by both CsA and rhodamine 123. This result strongly supports our theory that P-gp functions in the secretory transport of loperamide across the intestine.

The closed loop experiment showed that loperamide absorption is not diminished by the addition of P-gp substrates or inhibitors, despite the fact that loperamide accumulation in the intestinal mucosa was clearly influenced by the P-gp inhibitors in the inhibition study. This result may be attributable to its rapid absorption (80% in 15 min), which may be explained by its high lipophilicity. We reported that P-gp-mediated flux may be masked by passive permeation for compounds with high lipophilicity [35]. Therefore, P-gp was not found to contribute to the absorption of loperamide in this experiment. Together, these results strongly suggest that P-gp pumps loperamide out into the small intestinal lumen.

In addition, in all the experiments of rhodamine 123, addition of loperamide inhibited the secretion of rhodamine 123, suggesting that loperamide interacts with P-gp in the small intestine to some extent.

As for morphine, its transport rate was slower than that of loperamide in the rat small intestinal tissues mounted in an Ussing-type chamber and no secretory-directed transport was observed. As expected, addition of a P-gp substrate or inhibitor did not affect the transport of morphine. In the Caco-2 cells of the Transwell method, we observed significant secretory-directed transport of morphine. However, the secretory ratio was much lower for morphine than for loperamide (1.5 vs. 11.3). This result indicates that the morphine transport in Caco-2 cells is affected by P-gp, but the degree of contribution of P-gp to morphine transport is smaller than that for loperamide.

In the intestinal loop method, morphine disappearance and accumulation in the intestinal tissue were not affected upon the addition of a P-gp substrate or inhibitor with an exception of an increase in the accumulation of morphine. This increase, though statistically significant, was negligible.

In conclusion, transports of both morphine and loperamide are secretory-directed in the small intestine. P-gp partially contributes to this secretory-directed transport. It is suggested that P-gp plays a greater role in the transport of loperamide than in that of morphine, probably owing to their differential affinity for P-gp.

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