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TRANSPORT AND METABOLISM OF DELTA SLEEP-INDUCING PEPTIDE IN CULTURED HUMAN INTESTINAL EPITHELIAL CELL MONOLAYERS

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ABSTRACT:

A cultured human intestinal epithelial (Caco-2) cell monolayer was used to study the transport and metabolism of delta sleep-inducing peptide [DSIP (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu)]. DSIP is of interest because it has been reported to be capable of permeating biological barriers (e.g. blood-brain barrier), and this property has been related to its solution conformation. When applied to the apical (AP) side of Caco-2 cell monolayers, DSIP was rapidly metabolized (8.2 ± 1.1% remaining after a 2-hr incubation), affording Trp as the major metabolite and Trp-Ala as a minor metabolite. When DSIP was added to the basolateral (BL) side of the monolayer, the same metabolites were detected, but the peptide was more stable (70.6 ± 3.0% remaining after a 2-hr incubation). Inclusion of bestatin, an inhibitor of aminopeptidases, at concentrations up to 0.29 mM with DSIP on the AP side of the Caco-2 cell monolayer increased the stability of the peptide only slightly but dramatically altered the distribution of the metabolites (Trp-Ala became the major metabolite, and Trp became the minor metabolite). Inclusion of other aminopeptidase inhibitors (e.g. amastatin, puromycin) alone, dipeptidylpeptidase IV inhibitors (e.g. diprotin A, Gly-Pro) alone, inhibitors of proteases that require heavy metals for proper activity (e.g. EDTA, 1,10-phenanthroline) alone, or cysteine protease inhibitors (e.g. leupeptin) alone did not lead to significant stabilization of the peptide. However, inclusion of a combination of 0.29 mM bestatin and 1 mM diprotin A with DSIP on the AP side of the monolayers resulted in a substantial increase in the stability of the peptide (83.2 ± 3.7% remaining after a 2-hr incubation). However, under these conditions, a new metabolite (Trp-Ala-Gly-Gly-Asp-Ala-Ser) was observed with a formation that could be inhibited by inclusion of 1 mM captopril, an inhibitor of peptidyl dipeptidase A. Therefore, the stability of DSIP could be further increased (95.1 ± 1.6% remaining after a 2-hr incubation) by incubating the peptide with 0.29 mM bestatin, 1 mM diprotin A, and 1 mM captopril. However, even when the major metabolic pathways were inhibited on the AP side of the cell monolayer, no DSIP was detected on the BL side of a Caco-2 cell monolayer. These results suggest that a yet unidentified metabolic pathway is preventing the AP-to-BL flux of DSIP or that DSIP has lower "intrinsic" ability to permeate across cultured intestinal epithelial cells than across cultured brain endothelial cells, a cell culture model of the blood-brain barrier.

DSIP,[1] which is an endogenous nonapeptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu), has been reported to be useful in the treatment of insomnia (1), pain (2), and the symptoms of withdrawal from alcohol and opiates (3). DSIP has also been shown to influence the activity of the hypothalamic-pituitary-adrenal axis (4). In addition to its unique spectrum of pharmacological effects, DSIP has also been of interest because of its reported ability to permeate biological membranes. Despite its unfavorable physicochemical properties [*e.g.*: mol wt 849 daltons, log $PC_{octanol/saline} = -2.0$ (5), −2 charge at physiological pH], DSIP has been reported to permeate the blood-brain barrier both *in vivo* (5–9) and *in vitro* (10) as well as the intestinal mucosa of unweaned rats (11).

Studies by Mikhaleva *et al.* (12) and Nabiev *et al.* (13) in the early 1980s suggested that the ability of DSIP to permeate membranes may be related to a unique semicircular or pseudocyclic conformation that allows the side chains of the C- and N-terminal residues to be close to each other in space. The polar side chains of the peptide (*e.g.* Asp^5, Ser^7, and Glu^9) were proposed to be arranged such that they were folded into the interior of the looped structure, imparting a very lipophilic exterior surface to DSIP. Recently, our laboratory, using a variety of modern spectroscopic techniques, characterized a different set of solution structures for DSIP (14). In contrast to the structures proposed by Mikhaleva *et al.* (12) and Nabiev *et al.* (13), our results suggest that DSIP exists in dynamic equilibrium between unfolded and folded structures (two β-turn-rich regions in water and a helix-like structure in trifluoroethanol). On the basis of molecular modeling simulations, it appears that these conformational features impart compactness and amphiphilicity to DSIP (14). It was hypothesized that these structural features might contribute to the ability of DSIP to cross biological membranes.

Considering the reported ability of DSIP to permeate the intestinal mucosa of unweaned rats (11) and the high level of interest in oral peptide delivery (15–17), we decided to investigate the transport and metabolism of this nonapeptide using a cell culture model (Caco-2) of the intestinal mucosa. Caco-2 cells, which spontaneously undergo differentiation into confluent monolayers (18), have been shown to exhibit both the physical and the metabolic barrier properties of intestinal mucosal cells to peptides (19–23).

In this study, we have shown that the Caco-2 cell monolayer is both a metabolic (*e.g.* peptidases) and a physical (*e.g.* tight cellular junctions) barrier to the transcellular transport of DSIP. Surprisingly, it was observed that even when the major pathways of DSIP metabolism were inhibited, this peptide was still unable to permeate Caco-2

[1] Abbreviations used are: DSIP, delta sleep-inducing peptide; AP, apical; BL, basolateral; BMECs, brain microvessel endothelial cells.

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monolayers. These results are discussed in light of the unique conformational structure of the peptide and its reported ability to permeate the blood-brain barrier.

Materials and Methods

Chemicals. DSIP, amastatin, bestatin, captopril, EDTA, Gly-Pro, leupeptin, 1,10-*o*-phenanthroline, puromycin, Trp-Ala, sodium azide, 2-deoxyglucose, Trp, and Hanks' balanced salt solution (powder) were purchased from Sigma (St. Louis, MO). [^{3}H]-Mannitol was purchased from American Radiolabeled, Incorporated (St. Louis, MO). Trifluoroacetic acid was purchased from Pierce (Rockford, IL), and Transwells were purchased from Costar (Boston, MA). The suppliers of the reagents to culture Caco-2 cells were as described previously (18).

Caco-2 Cell Culture. Caco-2 cells were cultured as described previously (18). Caco-2 cells were grown in tissue culture flasks to 80% confluency and then trypsinized with 0.25% trypsin in phosphate-buffered saline containing 0.02% EDTA at a split ratio of 1:3 for passaging. For transport and metabolism experiments, cells were seeded on collagen-coated membranes in Transwells. Cells used were between passages 23 and 55. The medium was changed every other day during the first week after seeding and every day thereafter. [^{3}H]-Mannitol flux was used to verify the integrity of the cell monolayers. Typical [^{3}H]-mannitol flux values were less than 0.25%/cm^2/hr.

Transport and Metabolism Experiments. Caco-2 monolayers (from 21 to 28 days old) were rinsed three times with Hanks' balanced salt solution containing 25 mM glucose and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonate (pH 7.35, incubation buffer). The test solution (containing the peptide in the presence and absence of peptidase inhibitors in the incubation buffer) was added to the donor side, and incubation buffer was added to the receiver side (volumes of the AP and BL sides were 1.5 and 2.5 ml, respectively). Aliquots were taken from the donor (10 μl) and receiver (100 μl) sides at intervals of 0, 5, 15, 30, 60, 90, and 120 min after adding the test solution to the donor side. After adding 90 μl of incubation buffer to the samples from the donor side (dilution 1:10), all samples were diluted with 100 μl of HPLC mobile phase A. The amount (10 μl) taken from the donor side was considered to be negligible; the amount withdrawn from the receiver side (100 μl) was considered significant and was replaced with fresh incubation buffer. This dilution effect was corrected for in subsequent calculations. Samples were kept in the freezer (−20°C) until analysis.

Effect of pH, Temperature, and Metabolic Inhibitors. Unless otherwise stated, experiments were performed at 37°C, and the pH of the transport buffer was 7.4. To study the active transport of the dipeptide Trp-Ala by the dipeptide carrier, experimental conditions were changed. For example, experiments were performed at 4°C or after adjusting the pH of the transport buffer on the AP side to 6.0. To study the effect of metabolic inhibitors on the transport of Trp-Ala, the AP side of the cell monolayers was preincubated for 30 min with incubation buffer containing 2-deoxyglucose (50 mM) and sodium azide (5 mM).

HPLC Assay. The HPLC system used to detect DSIP and its Trp-containing metabolites (Trp-Ala-Gly-Gly-Asp-Ala-Ser, Trp-Ala, and Trp) consisted of a Shimadzu LC 6A gradient pump system, a Shimadzu Sil 6A autoinjector, a Rainin Dynamax-300 A C-18 column (4.6 mm × 250 mm, 5 μm), and a Shimadzu RF-535 fluorescence detector interfaced with a Shimadzu C-R4A Chromatopac integrator system (Shimadzu Scientific Instruments, Columbia, MD). Mobile phase A consisted of 5% acetonitrile, 0.1% trifluoroacetic acid, and 94.9% H_2O. Mobile phase B consisted of 80% acetonitrile and 20% H_2O. The flow rate of the mobile phase was 1 ml/min. Separation was carried out with a linear gradient from 0 to 30% of mobile phase B over 16 min, followed by a linear gradient over 2 min to 95% B, an isocratic state of 2 min, and a return to initial conditions over 2 min, followed by column reequilibration in solvent A for 8 min. The excitation wavelength of the fluorescence detector was set at 280 nm and the emission wavelength at 360 nm. The injected volume was 50 μl. For DSIP (1 μg/ml), the intraday, as well as the interday, variations were lower than 5%. Detector response was linear between 7.8 and 1000 ng/ml. The retention times (in min) were as follows: DSIP, 12.4; Trp-Ala-Gly-Gly-Asp-Ala-Ser, 11.9; Trp-Ala, 12.8; and Trp, 13.4.

Data Presentation. The results of peptide metabolism or transport studies are expressed as a percentage of the original amount of DSIP added to the donor compartment. The concentrations of the main metabolites of DSIP, Trp-Ala, and Trp, are expressed in ng/ml and were determined using standard curves for Trp-Ala and Trp prepared in incubation buffer/mobile phase A (50:50).

Results

When DSIP (concentration 11.7 μM) was included in the medium on the AP side of a Caco-2 cell monolayer, the peptide was rapidly metabolized, resulting in only 8.2 ± 1.1% DSIP remaining after a 2-hr incubation (fig. 1*A*, fig. 2*B*). Under these experimental conditions (application of DSIP to the AP side), no detectable level (<0.1% of the amount added to the AP side) of DSIP was measured on the BL side of the monolayer (data not shown). When DSIP (concentration 11.7 μM) was added to the BL side of the cell monolayer, the peptide was less rapidly metabolized, resulting in 70.6 ± 3.0% DSIP remaining after a 2-hr incubation (fig. 1*A*). Under these experimental conditions (application of DSIP to the BL side), no detectable level (<0.1% of the amount added to the BL side) of DSIP was measured on the AP side of the monolayer (data not shown). HPLC analysis of the medium on the donor side of the Caco-2 cell monolayer after a 2-hr incubation revealed that regardless of the side (AP or BL) of addition of DSIP, the major metabolite was Trp (fig. 1*B*, fig. 2*B*), and the minor metabolite was Trp-Ala (fig. 1*C*, fig. 2*B*). HPLC analysis of the medium on the receiver side (BL side after application of the DSIP on the AP side or AP side after application of the DSIP on the BL side) revealed only the presence of Trp (data not shown), which is presumably transported across the cell monolayer by the large neutral amino acid transporter, which has been characterized by Hu and Borchardt (24, 25) in Caco-2 cells. Based on the results shown in fig. 1 for the experiment involving the application of DSIP to the AP side of Caco-2 cell monolayers, approximately 8.6% of DSIP remained on the AP side, approximately 6.2% appeared as the Trp-Ala metabolite on the AP side, and approximately 59.9% and 10.3% appeared as the Trp metabolite on the AP and BL sides, respectively, after a 2-hr incubation. These initial DSIP metabolism results suggest that this peptide is being degraded in Caco-2 cells by aminopeptidases (E.C.3.4.11) and dipeptidyl peptidase IV (E.C.3.4.14.5) and that these enzymes are expressed at higher levels on the AP side than on the BL side of the Caco-2 cell monolayer.

Because the experimental results described above suggested that Caco-2 cell monolayers are both a physical and a metabolic barrier to the transport of DSIP, we attempted to circumvent the metabolic barrier by including peptidase inhibitors in the incubation medium to stabilize this peptide. Because aminopeptidases appeared to mediate the major pathway of DSIP metabolism in Caco-2 cells, we initially investigated the effects of bestatin, which has been reported to inhibit leucyl aminopeptidase (E.C.3.4.11.1), soluble arginyl aminopeptidase (E.C.3.4.11.6), soluble alanyl aminopeptidase (E.C.3.4.11.14), microsomal alanyl aminopeptidase (E.C.3.4.11.2), and tripeptide aminopeptidase (E.C.3.4.11.4) (26). As shown in fig. 3*B*, inclusion of bestatin with DSIP (11.7 μM) in the medium on the AP side of the cell monolayer produced a concentration-dependent reduction in the amount of Trp generated from hydrolysis of DSIP. However, even at the highest concentration of bestatin employed (0.29 mM), the stability of DSIP was only increased slightly (fig. 3*A*, fig. 2*C*). This inability of bestatin to significantly stabilize DSIP appears to result from the fact that when aminopeptidases are inhibited, the dipeptidyl peptidase IV pathway of DSIP metabolism becomes more significant. This conclusion is based on the observation of higher levels of Trp-Ala on both the AP side (figs. 2*C*, 3*C*) and the BL side (data not shown) of the DSIP-bestatin-treated cell monolayers.

The AP-to-BL transport of Trp-Ala, which is generated by the

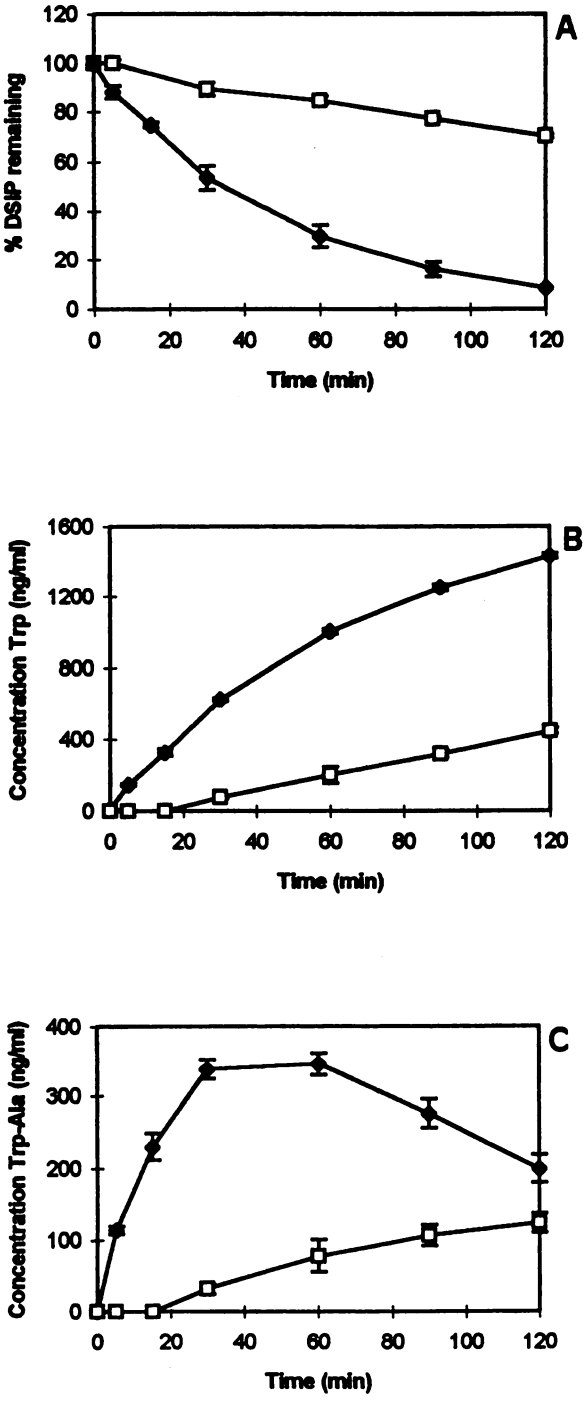

FIG. 1. *Time course of disappearance of DSIP and appearance of the major metabolites Trp and Trp-Ala on the donor side (AP or BL) of Caco-2 cell monolayers.*

DSIP (11.7 μM) was applied to the AP or BL side (donor side) of confluent Caco-2 cell monolayers (21 to 28 days old) in the incubation buffer. Aliquots (10 μl) were removed from the donor side at various time intervals, diluted with the incubation buffer (90 μl) and HPLC mobile phase A (100 μl), stored at −20°C, and analyzed by HPLC (see *Materials and Methods*). *Panel A*, disappearance of DSIP from AP (♦) or BL (□) side; *panel B*, appearance of Trp on AP (♦) or BL (□) side; and *panel C*, appearance of Trp-Ala on AP (♦) or BL (□) side. Data are the average of triplicate determinations ± SE.

metabolism of DSIP in the presence of bestatin, appears to be mediated by the di/tripeptide transporter reported to be present in Caco-2 cells (27–29). Evidence in support of this conclusion was provided by

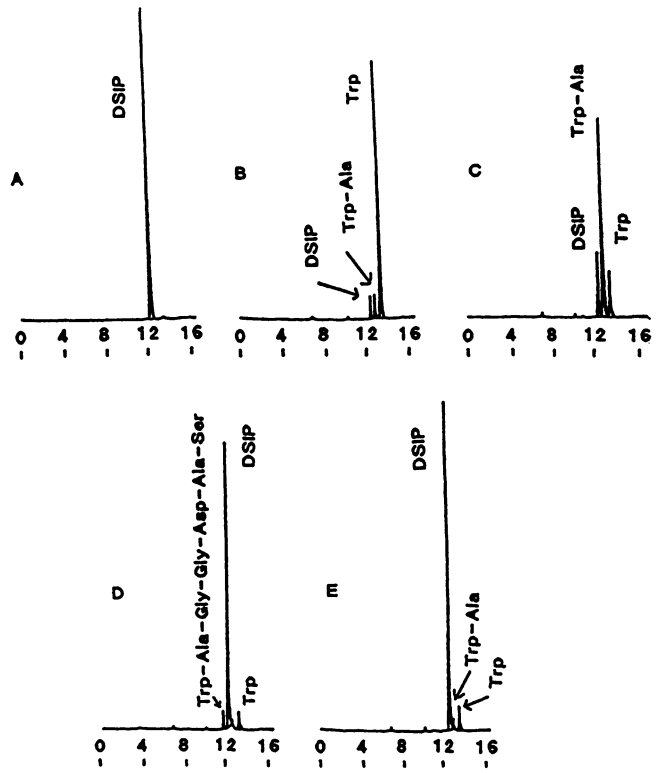

FIG. 2. *Typical HPLC chromatograms of DSIP applied to the AP side of Caco-2 cell monolayers in the absence or presence of peptidase inhibitors.*

DSIP (11.7 μM) (*panel A*) was applied to the AP side (donor side) of confluent Caco-2 cell monolayers (21 to 28 days old) in the absence (*panel B*) or the presence of 0.29 mM bestatin (*panel C*), 0.29 mM bestatin + 1 mM diprotin A (*panel D*), or 0.29 mM bestatin + 1 mM diprotin A + 1 mM captopril (*panel E*) in the incubation buffer. Aliquots were removed from the donor side after a 2-hr incubation and processed as described in *fig. 1*. Exact retention times for DSIP and its major metabolites are provided in *Materials and Methods*.

studying the AP-to-BL transport of Trp-Ala (36.3 μM) in the presence of bestatin (0.29 mM) in the donor side to minimize metabolism by aminopeptidases. Under these conditions, 1.56 ± 0.08% of the Trp-Ala was transported to the BL side of the monolayer after 2 hr. The AP-to-BL transport of Trp-Ala could be reduced to 0.70 ± 0.05% by inclusion of the metabolic inhibitors sodium azide (5 mM) and 2-deoxyglucose (50 mM). In addition, the AP-to-BL transport of Trp-Ala was reduced to 0.18 ± 0.04% by inclusion of Gly-Pro (10 mM) and to 0.21 ± 0.06% by performing these experiments at 4°C rather than 37°C. If the pH of the AP side was adjusted from 7.4 to 6.0, AP-to-BL transport of Trp-Ala increased to 3.7 ± 0.4%.

In further attempts to stabilize DSIP, the following protease inhibitors, which have been reported to inhibit the indicated enzymes, were also evaluated: amastatin, leucyl aminopeptidase (E.C.3.4.11.1) and glutamyl aminopeptidase (E.C.3.4.11.7); puromycin, soluble alanyl aminopeptidase (E.C.3.4.11.14); diprotin A and Gly-Pro, dipeptidyl peptidase IV (E.C.3.4.14.5); captopril, peptidyl dipeptidase A (E.C.3.4.15.1); EDTA and 1,10-phenanthroline, inhibitors of proteases that require heavy metals; and leupeptin, cysteine proteases (E.C.3.4.22) (16, 26). As shown in fig. 4*A*, all of these protease inhibitors, except for leupeptin, produced a slight stabilization of DSIP when included in the medium applied to the AP side of the cell monolayer. As expected, the aminopeptidase inhibitors (amastatin, puromycin) all significantly reduced the formation of Trp (fig. 4*B*). However, like bestatin, amastatin and puromycin also produced in-

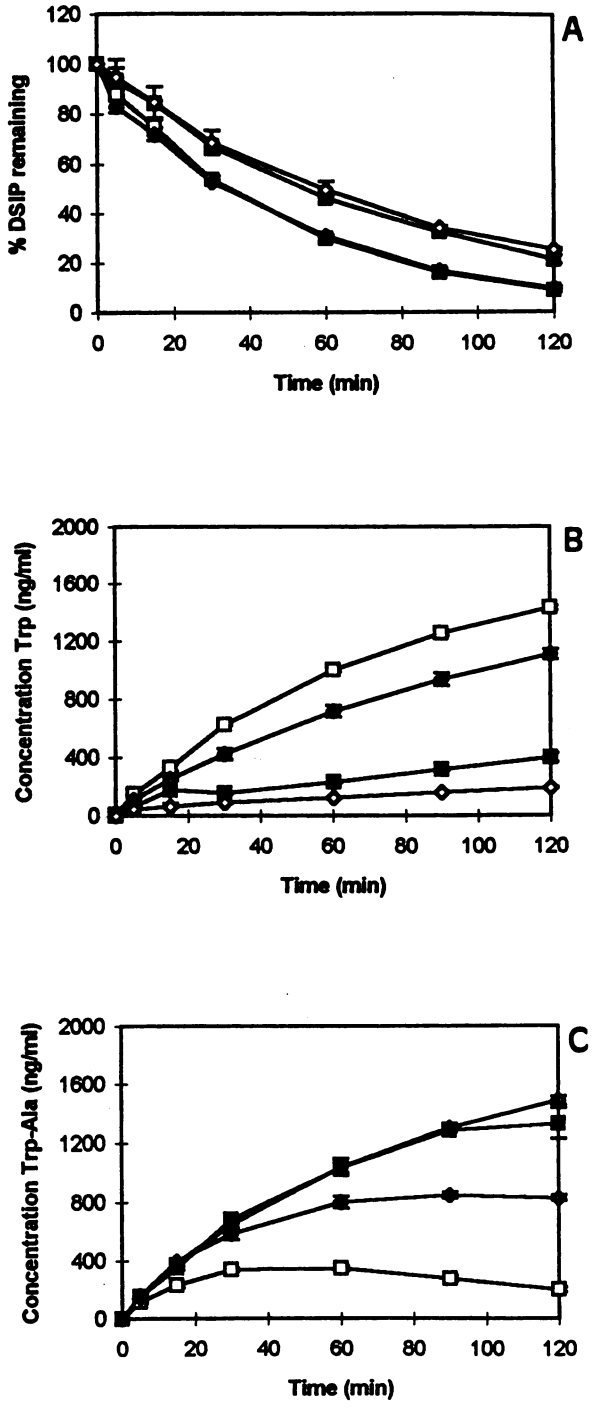

FIG. 3. *Effects of various concentrations of bestatin on the disappearance of DSIP and the appearance of the major metabolites Trp and Trp-Ala on the AP side of Caco-2 cell monolayers.*

DSIP (11.7 μM) in the absence (□) or the presence of various concentrations of bestatin (◇, 0.012 mM; ■, 0.058 mM; ⋆, 0.29 mM) was applied to the AP side (donor side) of confluent Caco-2 cell monolayers (21 to 28 days old) in the incubation buffer. Aliquots were removed from the donor side and processed as described in *fig. 1. Panel A*, disappearance of DSIP; *panel B*, appearance of Trp; *panel C*, appearance of Trp-Ala. Data are the averages of triplicate determinations ± SE.

creases in Trp-Ala (fig. 4*C*). The chelating agents (EDTA and 1,10-phenanthroline) also reduced the formation of Trp (fig. 4*B*). EDTA increased the formation of Trp-Ala; however, because of interferences in the HPLC chromatograms, a similar effect could not be verified for

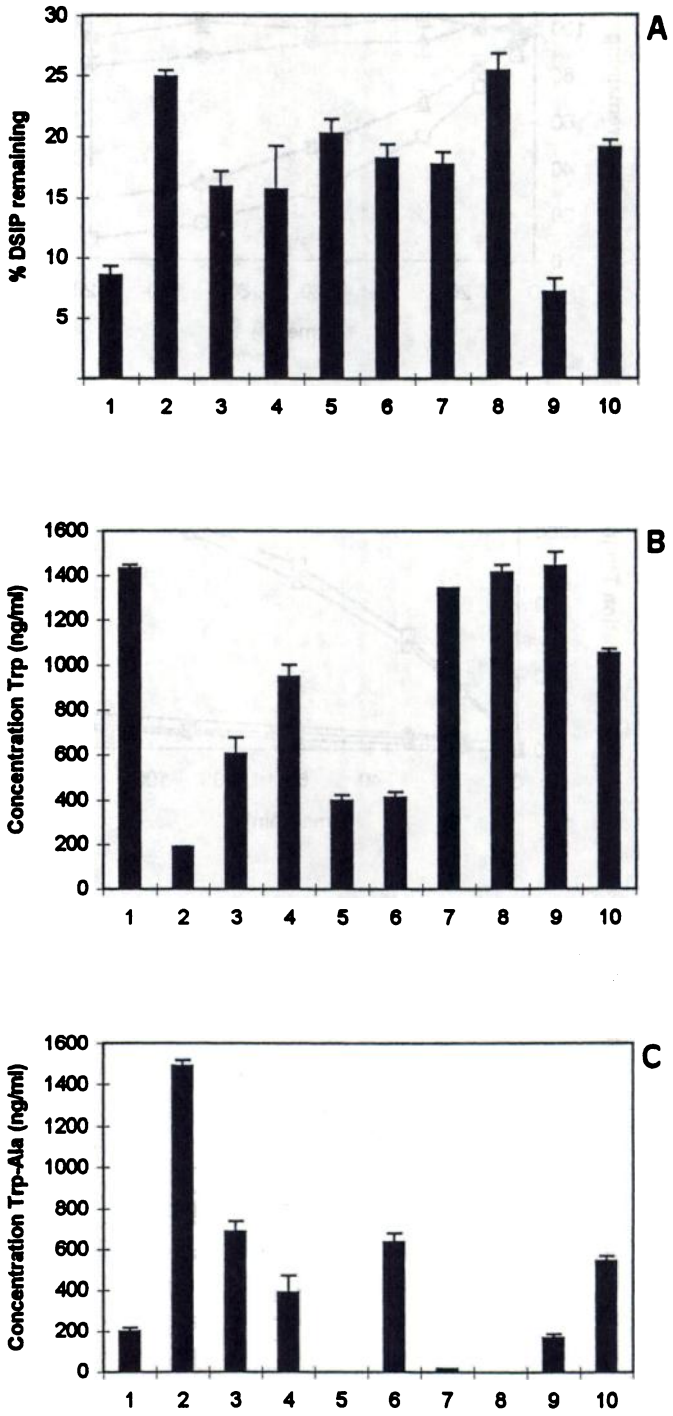

FIG. 4. *Effects of various peptidase inhibitors on the disappearance of DSIP and the appearance of the major metabolites Trp and Trp-Ala on the AP side of Caco-2 cell monolayers.*

DSIP (11.7 μM) in the absence (*1*) or the presence of various peptidase inhibitors (*2*, bestatin, 0.29 mM; *3*, amastatin, 0.2 mM; *4*, puromycin, 0.18 mM; *5*, 1,10-phenanthroline, 1.0 mM; *6*, EDTA, 2 mM; *7*, Gly-Pro, 29 mM; *8*, diprotin A, 1 mM; *9*, leupeptin, 2.1 μM; and *10*, captopril, 1 mM) was applied to the AP side (donor side) of confluent Caco-2 cell monolayers (21 to 28 days old) in the incubation buffer. Aliquots were removed from the donor side and processed as described in *fig. 1. Panel A*, disappearance of DSIP; *panel B*, appearance of Trp; *panel C*, appearance of Trp-Ala. Data are the averages of triplicate determinations ± SE.

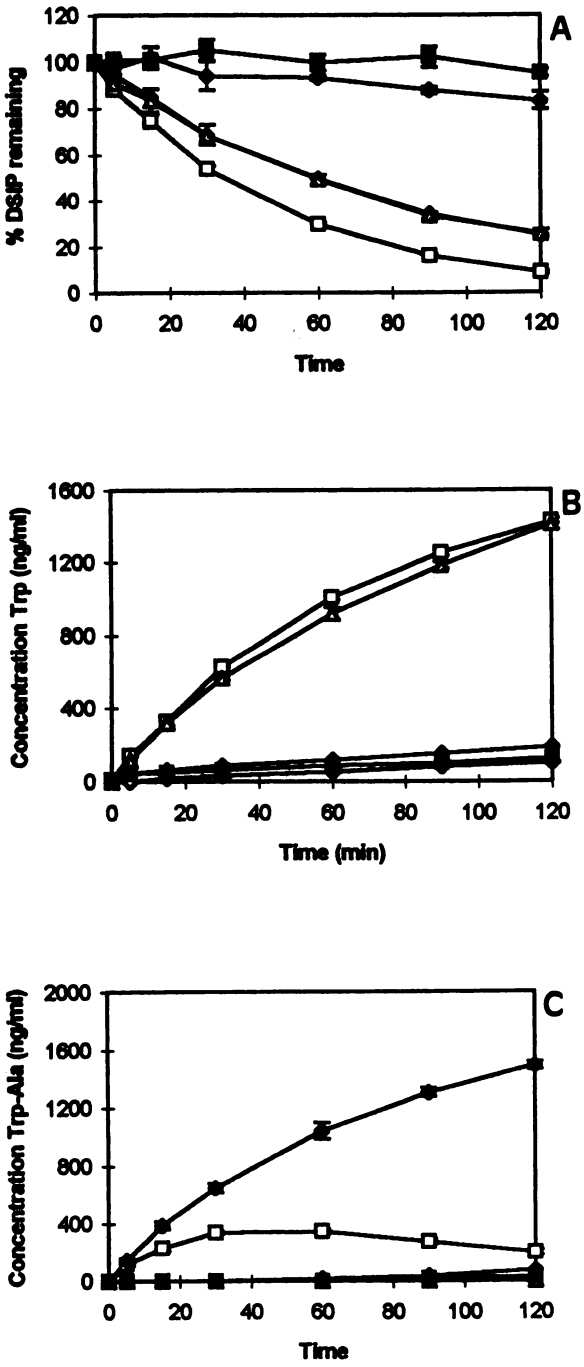

FIG. 5. *Effects of combinations of peptidase inhibitors on the disappearance of DSIP and the appearance of the major metabolites Trp and Trp-Ala on the AP side of Caco-2 cell monolayers.*

DSIP (11.7 μM) in the absence (□) or the presence of 0.29 mM bestatin (◇), 1 mM diprotin A (△), 0.29 mM bestatin + 1 mM diprotin A (◇) and 0.29 mM bestatin + 1 mM diprotin A + 1 mM captopril (■) was applied to the AP side (donor side) of confluent Caco-2 cell monolayers (21 to 28 days old) in the incubation buffer. Aliquots were removed from the donor side and processed as described in *fig. 1. Panel A*, disappearance of DSIP; *panel B*, appearance of Trp; *panel C*, appearance of Trp-Ala. Data are the average of triplicate determinations ± SE.

1,10-phenanthroline. Captopril, a peptidyl dipeptidase A inhibitor, also produced a slight decrease in the formation of Trp (fig. 4*B*) and a slight increase in the formation of Trp-Ala (fig. 4*C*). The dipeptidyl peptidase IV inhibitors diprotin A and Gly-Pro both produced a significant decrease in the formation of Trp-Ala (fig. 4*C*).

Because inclusion of inhibitors of aminopeptidases (*i.e.* bestatin, 0.29 mM) and dipeptidyl peptidase IV (*i.e.* diprotin A, 1 mM) separately did not significantly stabilize DSIP (figs. 2*C*, 3*A*, 4*A*), we evaluated a combination of bestatin (0.29 mM) and diprotin A (1 mM). Inclusion of a mixture of these inhibitors of aminopeptidases (bestatin) and dipeptidyl peptidase IV (diprotin A) substantially increased the stability of DSIP (figs. 2D, 5*A*), resulting in very low levels of Trp (figs. 2*D*, 5*B*) and Trp-Ala (figs. 2*D*, 5*C*). Under these conditions, 83.2 ± 3.7% DSIP remained on the AP side of the monolayer after a 2-hr incubation. However, under these experimental conditions, a new metabolite with a retention time of 11.9 min (fig. 2*D*) was detectable. This metabolite was shown to have the same retention time on HPLC as Trp-Ala-Gly-Gly-Asp-Ala-Ser (data not shown), suggesting metabolism by peptidyl dipeptidase A. The formation of this metabolite from DSIP could be completely inhibited by inclusion of captopril (1 mM), bestatin (0.29 mM), and diprotin A (1 mM) in the incubation mixture (fig. 2*E*). Under conditions where all three protease inhibitors are applied to the AP side of the monolayer, 95.1 ± 1.6% of DSIP could be recovered after a 2-hr incubation (fig. 5*A*).

Using the protocol developed to stabilize DSIP to peptidase metabolism (inclusion of bestatin, 0.29 mM; diprotin A, 1 mM; and captopril, 1 mM), AP-to-BL transport of this peptide across Caco-2 cell monolayers was studied. When DSIP (concentration 11.7 μM) was included with the protease inhibitors in the medium on the AP side of Caco-2 monolayers, no DSIP was detectable on the BL side after a 2-hr incubation. The sensitivity of the analytical methods employed indicated that less than 0.1% of DSIP was being transported per hr.

Discussion

The intestinal mucosa represents a significant barrier to the oral delivery of peptides into the systemic circulation (15–17). The barrier characteristics of intestinal mucosa cells arise because of the tight intercellular junctions, which limit paracellular flux of a peptide (physical barrier), and because of the abundance of peptidases associated with the brush border membrane and the cytoplasm (metabolic barrier), which rapidly metabolize peptides to their constituent amino acids (30–31). Therefore, it is of interest when a naturally occurring peptide is reported to exhibit the ability to permeate this physical and metabolic barrier.

One of these peptides is DSIP, which, in spite of its unfavorable physicochemical properties and the fact that it consists totally of L-amino acids, has been reported to permeate the blood-brain barrier both *in vivo* (5–9) and *in vitro* (10) and the intestinal mucosa of unweaned rats (11). This unexpected permeation of DSIP through biological barriers has been attributed to its ability to form unique solution conformations (12–14).

Because the intestinal permeability of DSIP has only been described in unweaned rats (11), which have an intestinal mucosa distinctly different both morphologically and biochemically from that observed in adult rat (30), we decided to investigate the transport and metabolism of this nonapeptide using a cell culture model of the intestinal mucosa (Caco-2 cells) (18–22). As expected, when DSIP was placed on the AP side of a Caco-2 cell monolayer, it was rapidly metabolized to form Trp as the major metabolite and Trp-Ala as the minor metabolite (fig. 1), suggesting the involvement of aminopeptidases and dipeptidyl peptidase IV, respectively. It was also observed that the rate of metabolism of DSIP was greater when applied to the AP side *vs.* the BL side of the monolayer (fig. 1), which is consistent with the AP localization of aminopeptidases and dipeptidyl peptidase

IV (32). It is interesting to note that when the aminopeptidases and dipeptidyl peptidase IV were inhibited by bestatin and diprotin A, respectively, the metabolism of DSIP by peptidyl dipeptidase A (E.C.3.4.15.1) was then observed (fig. 2*D*). The metabolism of DSIP by peptidyl dipeptidase A in Caco-2 cells can be inhibited by inclusion of captopril in the incubation mixture (fig. 2*E*).

It is interesting to compare the rate and the pathways of DSIP metabolism in Caco-2 cells with those observed in BMECs, an *in vitro* cell culture model of the blood-brain barrier. Raeissi and Audus (10) have reported a very slow metabolism ($t_{1/2}$ ~10 hr) of DSIP when applied on the AP side of BMEC monolayers with the major metabolite being Trp. The generation of Trp from DSIP suggests catalysis by aminopeptidases, which have been reported to be present in BMEC monolayers by Baranczyk-Kuzma and Audus (33). Recently, our laboratory has confirmed the involvement of aminopeptidases in the metabolism of DSIP in cultured BMECs.[2] However, we also observed the generation of significant quantities of several metabolites (Trp-Ala-Gly-Gly-Asp-Ala-Ser, Trp-Ala-Gly-Gly-Asp-Ala, Trp-Ala-Gly) of DSIP, which apparently arise from the action of peptidyl dipeptidase A. This hypothesis was confirmed when purified peptidyl dipeptidase A was shown to degrade DSIP to these same metabolites, and the production of these metabolites could be inhibited by captopril.[2] Two important differences exist between the metabolism of DSIP in Caco-2 cells and in BMECs. First, the metabolism in Caco-2 cells ($t_{1/2}$ ~35 min) is substantially faster than the metabolism in BMECs ($t_{1/2}$ ~10 hr). Second, in Caco-2 cells, metabolism is catalyzed by aminopeptidases (major pathway), dipeptidyl peptidase IV (minor pathway), and peptidyl dipeptidase A (very minor pathway). In contrast, in BMECs, both aminopeptidases and peptidyl dipeptidase A appear to contribute significantly to the metabolism of DSIP.

Because DSIP has been reported to be capable of permeating BMEC monolayers (10), we hypothesized that the lack of permeability of this peptide through Caco-2 monolayers may be a result of its higher rate of metabolism in this intestinal epithelial cell line. Therefore, we developed a protocol in this study that substantially stabilized DSIP (approximately 95% remaining after a 2-hr incubation) when this peptide was applied to the AP side of Caco-2 cell monolayers (figs. 2*E*, 5*A*). However, even when DSIP was stabilized to metabolism by aminopeptidases, dipeptidyl peptidase IV and peptidyl dipeptidase A in Caco-2 cells, no detectable quantities of this peptide were observed on the BL side of the cell monolayers after a 2-hr incubation.

It is possible that this impermeability of Caco-2 cells by DSIP could be due to a yet unidentified pathway of DSIP metabolism. This is possible, because the protocol described in this article does not totally stabilize DSIP to metabolism in Caco-2 cells (approximately 5% metabolism occurs during a 2-hr incubation). Therefore, although DSIP might have the correct physicochemical properties (12–14) to allow it to cross the Caco-2 cellular membranes, its ability to permeate these biological membranes may be limited by yet uncharacterized metabolic pathways in the cell membrane or the cytoplasm of these intestinal epithelial cells. If this hypothesis is correct, then these pathways of metabolism must play a less significant role in the metabolism of DSIP in BMECs, because this peptide is able to permeate both *in vivo* (5–9) and *in vitro* (10) through the blood-brain barrier.

With respect to the reported permeation of DSIP through the intestinal mucosa of unweaned rats (11), this observation may not be unexpected if one considers the morphological and biochemical differences between unweaned rat intestinal mucosa and adult rat intestinal mucosa (30). The intestinal mucosa in unweaned rat is known to be much more permeable to peptides and proteins than adult intestinal mucosa, resulting from the existence of less tight cellular junctions (thus increased paracellular flux of solutes), the lower expression of enzymes involved in peptide and protein metabolism, and higher vesicular trafficking of macromolecules (30). Thus, in unweaned rats, the intestinal mucosa may represent a less significant physical and metabolic barrier to DSIP than that observed in adult intestinal mucosa or Caco-2 cell monolayers.

In conclusion, the experimental results reported here illustrate that stabilization of a peptide to intestinal mucosal metabolism may require the use of a "cocktail" of peptidase inhibitors. Alternatively, multiple structure changes in the peptide may be necessary to stabilize the molecule to intestinal mucosal metabolism. However, even stabilization of a peptide to metabolism does not ensure that it will be able to permeate the intestinal mucosa. Additional structural changes may be necessary to provide the molecule with the optimal physicochemical characteristics necessary to ensure membrane permeability.

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[2] P. F. Augustijns and R. T. Borchardt, unpublished observation.

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