Different Properties of the Paracellular Pathway Account for the Regional Small Intestinal Permeability to the Peptide Desmopressin

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Abstract □ The regional small intestinal permeability to the vasopressin analogue desmopressin (dDAVP) was further characterized in proximal jejunal and distal (ileocecal) segments of the rat. Administration of the peptide to closed small intestinal loops confirmed the existence of regional absorption differences also *in vivo* in rats. Thus, the extent of absorption was about 4 times as high from the ileocecal part of the small intestine. The mucosal to serosal passage of dDAVP was studied in small intestinal segments from rats, mounted in Grass diffusion chambers. Increasing the mucosal concentration of dDAVP 100-fold had no effect on permeability coefficients (P_{app}) and the regional permeability distal:proximal ratio was maintained. Excessive amounts of unlabeled dDAVP to reduce the passage of [^{3}H]dDAVP had no effect either. These findings make the existence of an active, receptor-mediated transport mechanism unlikely, and dDAVP probably does not affect its own transport rate. The higher ileocecal permeability could either be due to the presence of more permeable or dynamic pores in this region, where the epithelial surface area is smaller, or to an increased capacity for paracellular water flux. These results may have relevance for drug transport in the small intestine, where site-specific delivery of drug or enhancing agents may be optimized.

Introduction

The oral delivery of peptide and protein drugs is an important issue in pharmacotherapy.[1] Oral administration is the preferred route for most drugs. However, due to their size, susceptibility to enzymatic degradation, and, in most cases, low lipophilicity, peptides and proteins are prevented from entering the blood circulation in significant amounts. One way of improving the peptide absorption in the gastrointestinal tract would be to target the drug to regions where the enzymatic activity is low and the absorption could be increased. Such a region is the part of the distal small intestine at the ileocecal junction which showed a higher absorption than the proximal small intestine of the nonapeptide dDAVP in rabbits.[2] This was later observed also in isolated rat intestinal segments[3] where a higher permeability was found in the distal small intestine. Other peptides displaying regional intestinal absorption differences include cyclosporin,[4] insulin,[5] and metkephamide.[6] In addition specialized transport systems for bile salts and cobalamin are located in the distal small intestine.[7]

The present investigation was conducted in order to further elucidate the observed permeability differences in the rat small intestine to dDAVP with consideration of the permeation routes through the intestinal epithelium by incubating intestinal tissue in diffusion chambers.[8] The possibility of an active transport mechanism for dDAVP was investigated by different saturation experiments, whereas paracellular diffusion was modulated with cytochalasin D and high concentration of glucose, both claimed to act as openers of tight junctions. In addition, it was considered important to corroborate the *in vitro* findings by studying the regional small intestinal absorption *in situ* in this species.

Materials and Methods

Chemicals—1-Desamino-8-D-Arginine-vasopressin (dDAVP, desmopressin) was obtained from Ferring Pharmaceuticals, Malmö, Sweden. The chromatographic purity of the peptide was >98%. [^{3}H]dDAVP (specific activity 16 mCi/μmol) was obtained from Axis Research AS (Oslo, Norway). [^{14}C]Mannitol (specific activity 2.1 GBg/mmol) was purchased from DuPont (Dreieich, Germany). Cytochalasin D was obtained from Sigma (St. Louis, MO).

Animals—Male rats of the Sprague-Dawley strain (Møllegaard, Skensved, Denmark), weighing 350–450 g, were kept on chopped wood bedding in polycarbonate cages under a 12 h day–night rhythm at 20 ± 2 °C, at a relative humidity of 50 ± 10%. The rats had free access to a rat chow (R3, Ewos, Södertälje, Sweden) and tap water.

Absorption Experiment in Situ—Food was withdrawn in the afternoon, and the experiments were started around 10 a.m. the following day. For repeated blood sampling, the rats were catheterized using silicon tubing (0.51 × 0.95 mm, Silastic, Dow Corning, Midland, MI) in the right jugular vein under Ketamine (Ketalar 50 mg kg^{-1} body wt, Parke-Davis, Barcelona, Span) anaesthesia and azaperone (Stresnil 0.13 mL/animal, Janssen, Beerie, Belgium). A midline abdominal incision was made and the small intestine was clamped in two places by ligation, providing either a proximal or a distal small intestinal loop. One ligature was placed in the proximal small intestine distal to the ligament of Treitz and the other 15 cm further down. In the distal ileum one ligature was placed proximal to the ileocecal junction and the other 15 cm further up. The peptide was administered (1 μmmol kg^{-1}) into the loop with a fine needle in a volume of 0.8 mL. Care was taken to avoid bleeding. Four rats were administered each route. Blood samples (0.5 mL) were taken at 0, 15, 30, 60, 90, and 120 min and replaced with 1 mL of isotonic saline. After the experiments the intestinal loops were examined for injury. One rat in each group had to be excluded while the others appeared macroscopically intact.

Permeability Experiments in Vitro—The experiments were commenced around 10 a.m. when the animals were anaesthetized using ether. An abdominal incision was performed and two 15 cm long segments, one proximal, taken 5 cm distal to the pylorus, and one distal, taken 5 cm proximal to the caecum, were removed. The segments were immediately immersed in room-temperature, modified Krebs–Ringer buffer, pH 7.4, (110.0 mM NaCl, 3.0 mM $CaCO_3$, 5.5 mM KCl, 1.4 mM KH_2PO_4, 29.0 mM $NaHCO_3$, 5.7 mM sodium pyruvate, 7.0 mM sodium fumarate, 5.7 mM sodium glutamate, and 13.4 mM glucose) oxygenated with carbogen (95% O_2 and 5% CO_2).

Each segment was divided in three pieces and was cut along the mesenterial border and mounted in diffusion chambers[8] (Precision Instrument Design, Los Altos, CA) with an exposed intestinal area of 1.78 cm^2. In this species we avoided stripping off the mucosa as the underlying tissue offered very little resistance to permeation.[10] The mucosal and serosal reservoirs were filled with 5 mL of buffer and were continuously oxygenated and gas-lift circulated at a temperature of 37 °C. Under these conditions the tissue preparations were considered viable for a 2-h period as shown by histological examination and active transport of 3-O-methyl glucose.[9,10] At the beginning of the incubations (t = 0), within 25 min from the induction of anaesthesia, the buffer at the mucosal side was exchanged with 5 mL of buffer containing the test substance. Thereafter aliquot samples were removed from the serosal side at 30, 60, 90, and 120 min with replacement with an equal volume.

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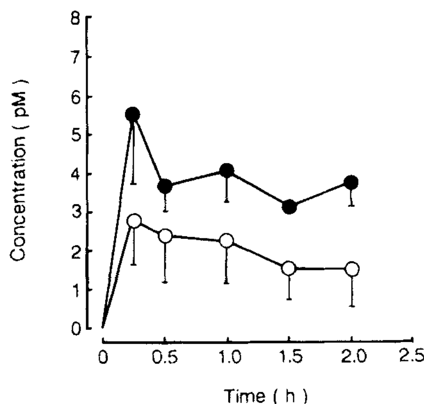

Figure 1—Time–plasma concentration curves for dDAVP after administration of 1 μmol kg^{-1} into proximal (open circles) and distal (filled circles) small intestinal loops. The curves were significantly different ($F = 4.55$, $p = 0.0026$). Values are shown as means ± SD ($N = 3$).

In the initial experiment dDAVP was added to mucosal reservoir at concentrations of 1, 10, and 100 μM. In the second experiment the permeation of [^{3}H]dDAVP (11.7 nmol, 1.84 μCi/mL) was examined in the presence on the mucosal side of 100 μM unlabeled peptide (ratio dDAVP:[^{3}H]dDAVP 1:10 000).

To assess the effects on tight junction permeability, cytochalasin D was added to the mucosal reservoir in a concentration of 10 μg/mL. [^{14}C]Mannitol and dDAVP were used as permeability markers. In another series of experiments, glucose (125 mM) was used as the permeability enhancing agent. The effect of increasing the osmolarity on the mucosal side by 330 mosm using polyethylene glycol 600 (PEG600, Sigma) was investigated for dDAVP.

Analyses—dDAVP was measured using a specific radioimmunoassay described in detail previously.[11] The limit of detection was 1 pM of peptide. [^{3}H]dDAVP and [^{14}C]mannitol were measured by liquid scintillation spectrometry (Rackbeta 1217, LKB, Bromma, Sweden). Buffer osmolarity was measured by freezing-point depression (Advanced Instruments Inc.).

Calculations—The apparent permeation coefficient (P_{app}) was calculated using the formula

$$P_{app}(\text{cm s}^{-1}) = (dc/dt)V(C_0A)^{-1}$$

where dc/dt represents the change of receiver reservoir concentration between 60 and 120 min (mol L^{-1} s^{-1}), V the volume in the chamber (L), C_0 the initial marker concentration in the donor reservoir (mol L^{-1}), and A the area of exposed intestine (cm^2). The withdrawal of 1 mL of reservoir fluid and refilling with 1 mL of buffer was compensated for in the calculations. AUC values were calculated using the trapezoidal rule by extrapolating the curves to the X-axis (Software; MK Model, Biosoft, UK). The extent of absorption (F) was calculated using a clearance (Cl$_p$) value for dDAVP of 0.474 L kg^{-1} h^{-1} (12) by the relationship:

$$F(\%) = \frac{\text{Cl}_p \times \text{AUC}}{\text{dose}} \times 100$$

Statistical comparisons between passage curves were performed by analysis of variance (ANOVA) and nonparametrically by the Mann Whitney U-test. A p-value of less than 0.05 was considered significant.

Results

The absorption of dDAVP was determined after intralumenal administration in closed intestinal loops from different regions of the small intestine. In the 2-h period following peptide administration a 2-fold higher absorption was observed from the distal ileal loops (Figure 1). The time–concentration curves were significantly different from each other ($p = 0.0026$). The slopes of the absorption phases show that there are absorption rate differences between the regions.

Table 1—Permeation Coefficients (P_{app}) for dDAVP Obtained from Small Intestinal Incubations in Diffusion Chambers[a]

Mucosal Concentration (M)	P_{app} (cm s^{-1} × 10^{-6}) Proximal	Distal	Mean Ratio
1 × 10^{-6}	2.97 ± 2.25	13.68 ± 5.59	4.6
1 × 10^{-5}	3.80 ± 1.52	6.93 ± 1.23	1.8
1 × 10^{-4}	3.93 ± 2.12	8.68 ± 4.25	2.2

[a] Results are given as means ± SD ($N = 9$). Differences between P_{app} values were evaluated by two-way ANOVA. There were no significant differences in P_{app} values between different dDAVP concentrations, while the distal:proximal differences were all significant ($p < 0.05$).

The AUC values for the proximal and distal time–concentration curves were 8.06 ± 3.9 and 38.1 ± 9.5 nmol h L^{-1}, respectively, and the extent of absorption 0.38 ± 0.18 and 1.81 ± 0.45%.

Isolated segments were mounted in diffusion chambers in order to further study the regional small intestinal permeability of dDAVP. Adding increasing concentrations of dDAVP to the mucosal (donor) reservoir resulted in similar P_{app} values for the intestinal segments (Table 1). The permeation of dDAVP was about twice as high across distal ileal segments compared with proximal and correlated with the mucosal concentrations (Figure 2). The regional distal:proximal permeability difference was maintained. The permeation of [^{3}H]dDAVP was not affected by excess amounts (1:10 000) of unlabeled peptide (Figure 2).

The presence on the mucosal side of cytochalasin D increased the permeability to dDAVP in both regions (Figure 3). For dDAVP the increase was 3.1-fold ($p = 0.0019$) in proximal and 1.36-fold ($p = 0.0081$) in distal segments. The corresponding effect on the permeation of [^{14}C]mannitol was almost 2-fold ($p = 0.0030$) in proximal but not observed in distal segments. When instead 125 mM glucose was added on the mucosal side the permeation of dDAVP increased 2.3-fold ($p = 0.0036$) in proximal and 1.7-fold ($p = 0.0201$) in distal segments (Figure 3). The permeation of [^{14}C]mannitol, on the other hand, increased 1.4-fold ($p = 0.0201$) in proximal and was unchanged in distal segments. Increasing the osmolarity on the mucosal side reduced the transport of dDAVP in distal ($p < 0.02$) but not in proximal segments (Figure 4).

Discussion

This study was conducted in order to characterize the previously reported regional differences within the small intestine regarding both *in vivo*[2] absorption and permeability *in vitro*.[3] Since previous *in vivo* experiments were performed in rabbits and the *in vitro* experiments in rats, there were no data on the *in vivo*:*in vitro* correlation in one species. The present experiments, however, demonstrate that the higher distal ileal permeability during a 2-h period observed in diffusion chambers correlates with a higher absorption of dDAVP from isolated distal ileal loops in the same period of time. It is apparent that the absorption rate for dDAVP is higher in the distal intestine, as was observed earlier in rabbits.[2] These results further justify the use of intestinal segments mounted in diffusion chambers as a valid *in vitro* model. It cannot, however, be concluded that the present observations extend to animal species other than the rabbit and rat.

We previously showed that the extent of dDAVP absorption, after intragastric feeding in rats, was below 0.1%.[12] Corrected for dose, the absorption was higher when the peptide was directly injected in closed intestinal loops, as in the present study. This could be attributed to either a lower luminal degradation or less mixing and adsorption of dDAVP to

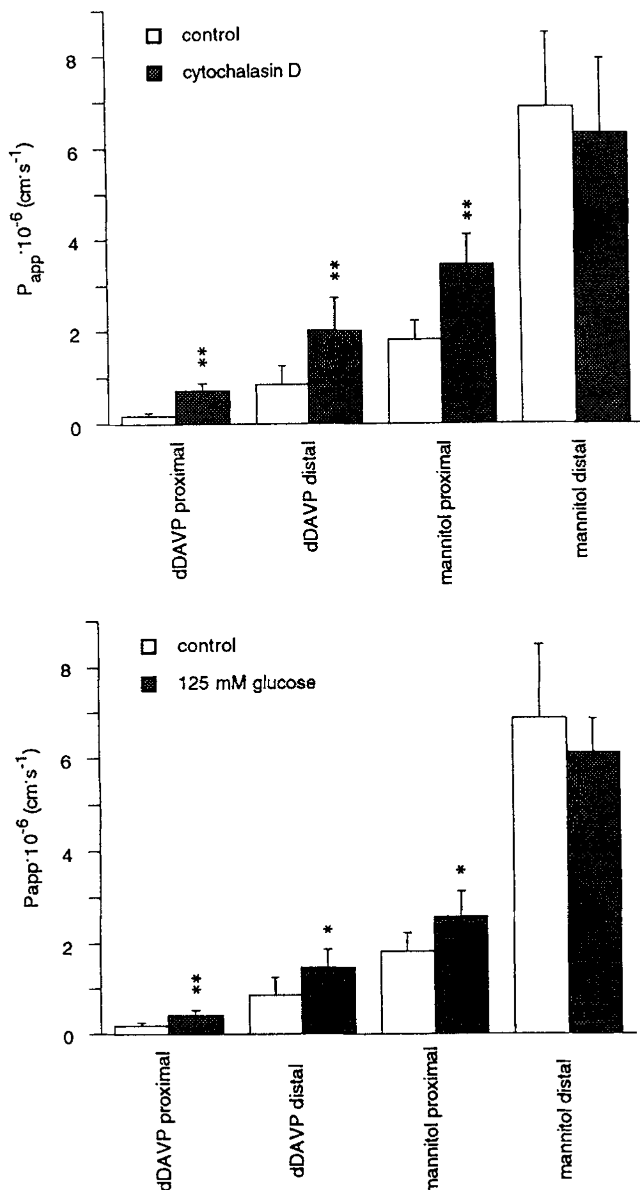

Figure 2—Permeation coefficients (P_{app}) for dDAVP and [^{14}C]mannitol across proximal jejunal and distal ileal intestinal segments mounted in a diffusion chamber in the presence of (a) glucose and (b) cytochalasin D. Values are shown as mean ± SD (N = 9).

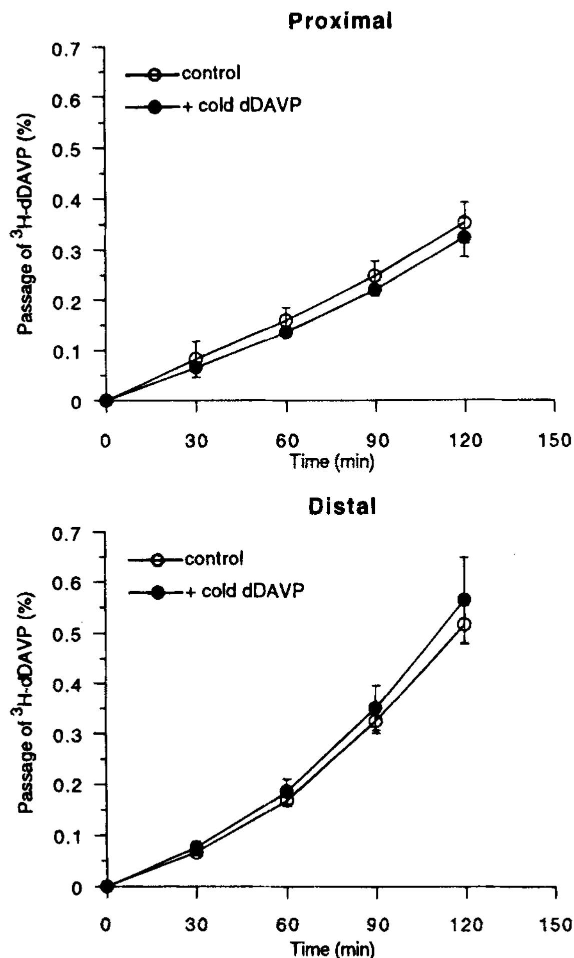

Figure 3—Mucosal to serosal transport of [^{3}H]dDAVP across proximal jejunal and distal ileal small intestinal segments mounted in a diffusion chamber with (open circles) and without (filled circles) the presence of excess amounts of unlabeled peptide (100 nM). Values are shown as means ± SD (N = 9).

intestinal contents. The proximal part of the small intestine has a larger surface area due to the presence of larger villi. Despite this, both absorption and permeability were highest in the distal part of the ileum. A number of possible explanations for this regional permeability difference can be suggested. There may be regional differences in proteolytic enzyme activity along the intestine, lumenal as well as mucosal.[13] A transcellular route through putative aqueous pores could exist in some part of the small intestine. It could also be that the extracellular (paracellular) transport route[14] shows different characteristics in different parts of the intestinal epithelium. The first of these possibilities, regional proteolytic activity, can be ruled out regarding dDAVP permeability *in vitro*, since this peptide was earlier shown to be stable in incubations with intestinal segments in rats[3,15] as well as with purified plasma membranes from various parts of human gastrointestinal tract.[16] The second possibility, presence of an active uptake mechanism, is highly unlikely according to the present results. We could not demonstrate any saturation of transmucosal transport by increasing the mucosal concentration of dDAVP or by inhibiting the mucosal to serosal transport of labeled peptide with excess amounts of unlabeled peptide. The recently cloned intestinal peptide transporter was not shown to transport peptides larger than four amino acids,[17] which does not give support to the existence of an active uptake mechanism for dDAVP. However, peptides may still be cotransported in other uptake systems.[18]

Our results suggest that the higher permeability to dDAVP in the distal small intestine could be ascribed to a more frequent occurrence of pores or pores that are larger in this region compared to the proximal part but also to regional differences in water flux in the small intestine. To increase paracellular permeability, two principles were used. Cytochalasins act on cytoskeletal elements (actin filaments) which are in contact with the occluding junctions at the cell surface. Contraction of these cytoskeletal proteins is believed to cause a widening of the occluding junctions.[19] Transcellular concentrative transport of glucose, on the other hand, provides the force for osmotic flow and at the same time triggers contraction of the perijunctional actomyosin, which in turn leads to an increased paracellular osmotic flow and solvent drag.[20] Both of these principles were used in the present study to assess the properties of the paracellular route at regions in the small intestine where paracellular permeability was found to differ. A stronger enhancement of the permeation was achieved in the proximal small intestine as shown by a diminished ratio between distal and proximal Papp

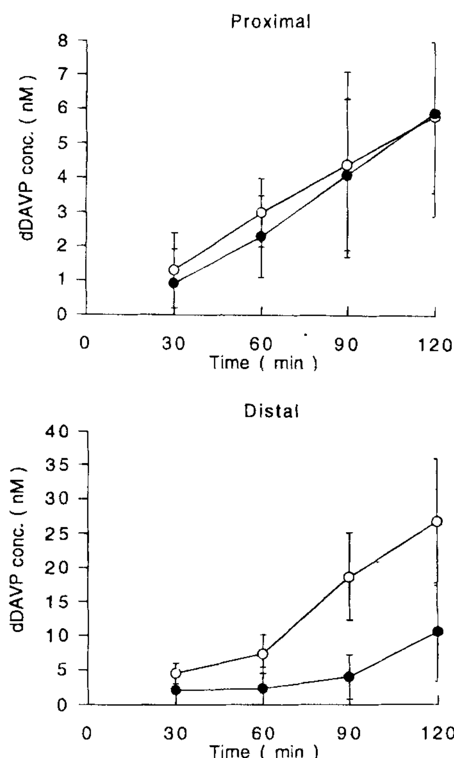

Figure 4—Mucosal to serosal transport of dDAVP across jejunal and distal ileal (ileocecal) small intestinal segments mounted in a diffusion chamber without (open circles) and with (filled circles) an increase osmolarity (660 mosm) induced by PEG 600. The normal Krebs–Ringer buffer osmolarity is 330 mosm. Values are shown as mean ± SD (N = 8–10).

values for both dDAVP and [^{14}C]mannitol. In the distal part the permeability increased for dDAVP but not for [^{14}C]-mannitol. It may be speculated that two populations of pores exist, a large population of small pores allowing mannitol passage and a small population of large pores allowing dDAVP passage. According to our results the relative number of large pores should be higher in the distal ileum. Both pore types are dilated in response to glucose and cytochalasin D. This dilatation affects the permeation of the larger more restricted molecule, dDAVP, more than [^{14}C]mannitol, suggesting that the pores in the distal part are larger or more "open" during normal conditions and do not restrict the permeation as much as in the proximal small intestine, as indicated by an unchanged [^{14}C]mannitol permeability in the distal part after cytochalasin D and glucose stimulation. The properties of intercellular pores are dependent on the number of tight junction strands circumscribing the apex of epithelial cells so that the larger the number of strands, the smaller the permeability. The dynamic properties of tight junctions may thus be a consequence of this morphological structure.[21] Another contributing factor could be that only charge-selective pores are dilated, which affects the positively charged dDAVP.[19] When a higher osmotic load was applied to the mucosal side according to Ma et al.,[22] the transport of dDAVP was unaffected in proximal and decreased in distal segments. This finding can be interpreted in two ways. It could mean that the intracellular space between distal enterocytes collapses, leading to a decrease in permeability,[23] or that the paracellular water flux may influence absorption in this part of the intestine. Our findings seemingly contradict current knowledge of intestinal permeability.[24,25] The choice of the most distal part of the ileum close to the ileocecal junction for permeability measurements might be important. Our earlier findings in rabbits[2] showed that the absorption of dDAVP was considerably lower in "mid"-ileum than in jejunum, increasing dramatically at the ileocecal junction and, again, decreasing in colon. Similar observations were made using everted sacs (Lundin et al., unpublished results).

In addition to the possible mechanisms of intestinal permeation of dDAVP, it is not known whether the peptide could exert some action on the epithelium itself, which has been the case for other peptides such as angiotensin II[26] and the synthetic PZ-peptide.[27] However, to date no receptors for vasopressin or dDAVP have been demonstrated in intestinal epithelial cells.

The present results show that small intestinal absorption can be improved by directing the peptide drug to a region where the permeability is higher and transit time prolonged. Efforts should therefore be directed to develop delivery systems that protect the peptide while at the same time prolonging contact with the absorption site. Although the intestinal absorption of most peptides is low, even a small absorption improvement could be important due to the generally high potency of these drugs.

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