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Stimulation by enkephalins of D-glucose absorption in rabbit ileum

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Abstract. In intact tissue, DAGO ([D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; 10^{-5} M; μ-ligand; addition on the serosal side) stimulated D-glucose absorption and D-glucose-dependent variations in short-circuit current ($\Delta I_{sc,glu}$); naloxone (10^{-6} M) antagonized these effects. DADLE ([D-Ala^2, D-Leu^5]enkephalin, mainly a δ-ligand; 10^{-5} M) and (pCl-Phe^4)-DPDPE ([D-pen^2, p-chloro-Phe^4, D-Pen^5]enkephalin, a more selective δ-ligand; 10^{-5} M) did not significantly stimulate $\Delta I_{sc,glu}$ (addition on the serosal side). In the absence of the muscularis and myenteric plexus or using intact tissue treated with tetrodotoxin (TTX; 3×10^{-7} M), DAGO was unable to increase $\Delta I_{sc,glu}$. Addition of DAGO to the mucosal side did not induce any variations in $\Delta I_{sc,glu}$. In conclusion, DAGO is able to increase D-glucose absorption by interacting with μ-receptors located in the myenteric plexus.

Key words. Enkephalins; myenteric plexus; glucose transport; short-circuit current.

Endogenous opioid peptides perform an important role in the intestine, especially in controlling motility, secretion and absorption [1–4]. Moreover, some pathological states [5], including diabetes [6], can modify the enkephalin concentration in the gastrointestinal tract.

Little is known at present about the effects of opioids on organic solute transport or their mechanism of action, although DAGO ([D-Ala^2, MePhe^4, Gly-ol^5]enkephalin) has recently been shown to inhibit intestinal absorption of L-valine in the rabbit [7]. We therefore investigated the effects that enkephalins might exert on the specific transport mechanisms of various organic solutes, and in particular their effect on intestinal absorption of D-glucose. On the basis of numerous in vitro studies using intestinal preparations from various animal species of the antisecretory effects of opioids [8–11], it can be concluded that these substances also act at a local intestinal level. Tetrodotoxin (TTX), a powerful neurotoxin that reduces the functional activity of the nervous plexa [12], can prevent the antisecretory effects of opioids in rabbit ileum [8] and mouse jejunum [11], which suggests that the effects of opioids on water transport depend on their interaction with intramural neurons. It should also be borne in mind that enkephalins [13, 14] and opioid receptors are present in the components of the enteric nervous system [15, 16]. We therefore tested whether the nervous plexa inside the intestinal wall play a part in regulating nutrient transport. The preliminary data support this hypothesis [7].

We also wished to establish whether opioids had any effect on the apical membrane of the enterocytes. In fact the exorphines, opioid peptides obtained from the digestion of some proteins present in food, can influence intestinal absorption on the mucosal side of the epithelium. β-casomorphines, for example, can influence short-circuit current [17, 18] and L-leucine transport [19], although they may reach the receptors on the serosal side through the junctional pathway.

Glucose absorption has been studied by analysing the variations in short-circuit current occurring after the addition of glucose (most of the glucose absorption

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through the apical membrane being Na^+-dependent), and directly measuring the net glucose fluxes. For our experiments we used DAGO, which acts on the μ-receptors, DADLE ([D-Ala^2, D-Leu^5]enkephalin) and (pCl-Phe^4)-DPDPE ([D-Pen^2, p-chloro-Phe^4, D-Pen^5]-enkephalin), which act mainly or more selectively on the δ-receptors. We chose the distal ileum because enkephalin receptors have mainly been identified in the ileum [3, 20, 21], and studies of the effect of opioids on epithelial transport largely refer to the ileum [7–10, 17]. In addition, the use of increases in short-circuit current (caused by the presence of luminal glucose) to evaluate Na^+-glucose cotransport in rabbit distal ileum is well established [22].

Materials and methods

Male New Zealand rabbits were killed by cervical dislocation, and a 5–10 cm segment of distal ileum was excised and placed in Krebs-Henseleit saline (mM concentrations: Na^+, 142.9; K^+, 5.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 127.7; HCO_3^-, 24.9; $H_2PO_4^-$, 1.2; SO_4^-, 1.2; pH 7.4). The salines were kept at 30 °C [7, 23] and bubbled with 95% O_2 and 5% CO_2 in both the electrophysiological and the everted sac experiments. In some experiments the serosa and muscularis were removed by blunt dissection.

Electrical measurements. The method was applied as reported above [7]. Briefly, the intestine, opened as a flat sheet, was mounted between the two halves of Ussing chambers (exposed area: 0.67 cm^2) and bathed on both sides with Krebs-Henseleit solution (5 ml on each side). V_{ms} (the transepithelial electrical potential difference) and I_{sc} (the short-circuit current) were measured with an automatic device made in our electronic workshop that subtracts the bathing fluid resistance to give a correct measurement of I_{sc}. Every 10 min, starting from the fifth minute after mounting, the initial luminal solution was replaced for 2.5 min with a similar solution in which 11 mM D-glucose replaced 5.5 mM NaCl so that osmolality was kept constant. D-glucose was not added to the serosal side, in order to favour the electrogenic component (Na^+ cotransported with glucose on the apical side) of the transepithelial transport. $\Delta V_{ms,glu}$ and $\Delta I_{sc,glu}$ represent the maximal increase due to the presence of glucose. In some experiments L-valine (20 mM) or 3-O-methyl-D-glucose (3-O-MG: 11 mM) was added to the luminal side instead of D-glucose, and $\Delta I_{sc,val}$ or $\Delta I_{sc,3\text{-}O\text{-}MG}$ represents the maximal increase of I_{sc} due to the presence of L-valine or 3-O-MG. The initial conditions (D-glucose- or L-valine-free Krebs-Henseleit solution) were restored by three washings. When used, saline containing DAGO, DADLE or (pCl-Phe^4)-DPDPE (10^{-5} M; Bachem, Switzerland) was present on the serosal or mucosal side at the beginning of the experiment (i.e. soon after the tissue was mounted). There are some experimental indications that ouabain, whose molecular weight is higher than that of the synthetic enkephalin we used, reaches the basolateral side of the enterocytes by diffusion (intact tissue) when added to the serosal incubation medium [24, 25]. The same thing should also happen in the case of the enkephalins used, as confirmed by our previous findings [7]. When naloxone (10^{-6} M: donated by Endo Laboratories, USA) or TTX (3×10^{-7} M: Fluka, Switzerland) was used, the tissue was kept in contact with the drugs for 5 min immediately after the animals were killed (i.e. during isolation, washing and mounting). This was done to pretreat the tissue without altering the usual time schedule.

Water, ion and D-glucose transport with everted sac preparation. Water, ion and D-glucose transport on the ileum were measured in everted sac preparations [7]. Water transport was evaluated following the dilution of an impermeable dye (0.07 mM phenol red, spectrophotometric determination; $\lambda = 505$ nm) added to the serosal side [25]. D-glucose was present at 11 mM on both sides of the tissue from the beginning of the experiment so that the net active transepithelial transport of D-glucose could be measured. The initial volumes were 2.5 and 100 ml on the serosal and luminal sides respectively; 10 min after the tissue was mounted (the time required to restore functional conditions after tissue isolation), 0.5 ml of saline was taken from the serosal side for measurement. Three identical samples of solution were taken at 30 min intervals. Enkephalin was added on the serosal side at the end of the first 30 min period with 20 µl of saline, to give a final concentration of 10^{-5} M. The calculation of the net fluxes took account of all volumes added or removed. The fluxes were calculated on the basis of the data obtained every 30 min, but are shown in the figures as moles cm^{-2} h^{-1}. The samples were deproteinized (by precipitation in 0.6 M trichloroacetic acid) and centrifuged. The serosal Na^+ and Cl^- concentrations were determined chemically with a flame photometer (model 943, Instruments Laboratory, Milan, Italy) and a chloridometer (Chloro-counter Mark II, Marius Instrument, Utrecht, Holland). The D-glucose concentration was determined spectrophotometrically by a colorimetric test (God-Perid Kit, Boehringer Mannheim GmbH, Germany).

Statistical analysis. Statistical significance was assessed by the ANOVA multiple range test in the case of the electrophysiological experiments; a sequential rejective multiple test was used for the experiments with everted intestinal sacs [26]. The data are reported as mean values S.E. with the number of animals (in brackets in the figures).

Results

The effects of enkephalin on transepithelial glucose transport. Under control conditions the $\Delta I_{sc,glu}$ values (fig. 1) showed a small spontaneous decrease over time, although the values obtained between 15 and 55 min did not differ significantly from each other. If, instead of using D-glucose, we added 3-O-MG, a non-metabolized sugar [27], we obtained $\Delta I_{sc,3\text{-}O\text{-}MG}$ values which did not differ significantly from the $\Delta I_{sc,glu}$ values (average value of $\Delta I_{sc,glu}$ between 15 and 55 min $= 27.1 \pm 2.2$ μA cm^{-2}, n = 46; $\Delta I_{sc,3\text{-}O\text{-}MG}$ at 5 min $= 36.4 \pm 2.9$ μA cm^{-2}, n = 10; average value of $\Delta I_{sc,3\text{-}O\text{-}MG}$ between 15 and 55 min $= 28.8 \pm 2.8$ μA cm^{-2}, n = 10). When DAGO (10^{-5} M) was present in the incubation medium on the serosal side, I_{sc} significantly decreased compared with the control, starting 25 min after treatment (for example, after 25 min the I_{sc} of the control was 41.2 ± 2.9 μA cm^{-2}, n = 46; after DAGO treatment the I_{sc} was 20.6 ± 2.8 μA cm^{-2}, n = 17, $p < 0.05$), which agreed with our earlier observations [7]. The $\Delta I_{sc,glu}$ values were significantly higher than those of the control starting from the fifteenth min after treatment (fig. 1). Naloxone (10^{-6} M) antagonized both the inhibitory effect on I_{sc} (as previously reported [7]) and the stimulatory effect on $\Delta I_{sc,glu}$ (fig. 1). The time courses of $\Delta V_{ms,glu}$ and $\Delta I_{sc,glu}$ were similar (for example, after 15 minutes $\Delta V_{ms,glu}$ increased from 1.5 ± 0.1 mV, n = 46 to 2.0 ± 0.1 mV n = 17 in the presence of DAGO). The addition of DAGO in electrophysiological experiments performed with 3-O-MG produced $\Delta I_{sc,3\text{-}O\text{-}MG}$ values which were not significantly different from those obtained for $\Delta I_{sc,glu}$ under the same conditions (the average value of $\Delta I_{sc,3\text{-}O\text{-}MG}$ between 15 and 55 min was 38.7 ± 3.5 μA cm^{-2}, n = 7, which was not significantly different from the $\Delta I_{sc,glu}$ value obtained in the same time range, 36.6 ± 2.8 μA cm^{-2}, n = 17, but differed significantly, $p < 0.05$, from the control values). The addition of DADLE or (pCl-Phe^4)-DPDPE (10^{-5} M) did not produce statistically significant effects on $\Delta I_{sc,glu}$ (fig. 1).

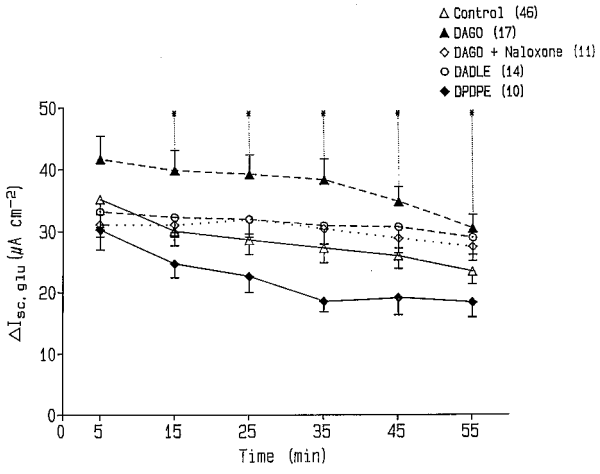

Figure 1. Intact tissue: effect of DAGO or DADLE or (pCl-Phe^4)-DPDPE (10^{-5} M; serosal side) or DAGO (10^{-5} M) plus naloxone (10^{-6} M) on D-glucose dependent variations in I_{sc} ($\Delta I_{sc,glu}$). The data points represent mean value ±S.E. Number of animals in brackets. *$p < 0.05$ compared with control.

On the basis of the results obtained with the short-circuit current technique, we measured transepithelial transport with the everted sac technique. Under control conditions, the net fluxes of H_2O, Na^+, Cl^- and D-glucose (fig. 2A, 2B) did not change to statistical significance during the three experiment periods. The addition of DAGO on the serosal side after the first period (10–40 min) caused a significant increase in net ion, water and D-glucose transport (fig. 2C, 2D) starting from the second period (paired data analysis). A statistically significant difference during the second period of DAGO treatment was observed when the treatment was compared with the control over the same periods with unpaired data.

Mechanism of action of enkephalins. When a tract of ileum deprived of serosa, muscle layers and myenteric plexus was used, the V_{ms} and $\Delta V_{ms,glu}$ values were similar to those observed in intact tissue. Both I_{sc} and $\Delta I_{sc,glu}$ increased as expected, since tissue resistance is reduced (by approx. 50%) [7]. The increase in I_{sc} and $\Delta I_{sc,glu}$ was foreseeable in view of the fact that I_{sc} and $\Delta I_{sc,glu}$ represent the electrogenic transepithelial active ion fluxes and the glucose-dependent increase in transepithelial Na^+ flux respectively in tissue deprived of muscle layers (in intact tissue, I_{sc} and $\Delta I_{sc,glu}$ merely represent values proportional to these electrogenic transepithelial active ion fluxes).

Treatment with DAGO (10^{-5} M) reduced I_{sc} by comparison with the control over a time course similar to that observed in intact tissue, in accordance with our earlier findings [7]. The $\Delta I_{sc,glu}$ values did not differ statistically from the $\Delta I_{sc,glu}$ values under control conditions, though a tendency to decrease was observed (fig. 3). We evaluated the effects of adding TTX alone (3×10^{-7} M) on $\Delta I_{sc,glu}$ in intact tissue (and the effects on $\Delta I_{sc,val}$ as a countercheck with reference to our earlier paper [7]); no significant variation emerged (fig. 4A, 4B). Conversely, when DAGO was present, the addition of TTX significantly antagonized the stimulatory effects on $\Delta I_{sc,glu}$, which proved statistically similar to the control values (fig. 4A). Similarly, TTX antagonized (fig. 4B) the inhibitory effects of DAGO on $\Delta I_{sc,val}$ [7]. In addition, the $\Delta I_{sc,glu}$ and $\Delta I_{sc,val}$ values obtained in the presence of DAGO plus TTX did not differ significantly from those measured in the presence of TTX alone.

We also investigated the effects of adding DAGO on the luminal side. $\Delta I_{sc,glu}$ and $\Delta I_{sc,val}$ did not vary to

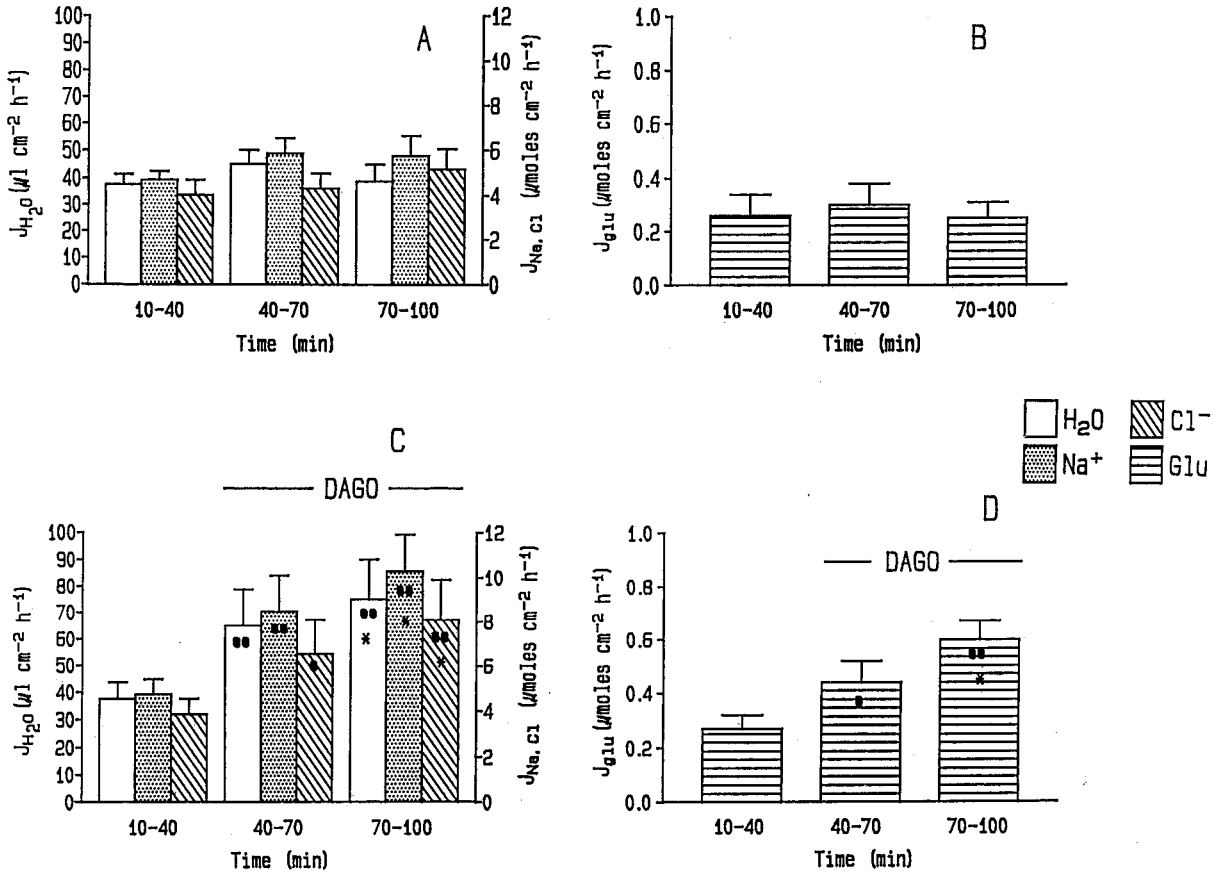

Figure 2. Intact tissue: net absorption of water, Na^+, Cl^- and D-glucose measured in the absence (*A*, *B*) or presence (*C*, *D*) of DAGO (10^{-5} M; addition after 40 min on the serosal side). D-glucose was present at the same concentration (11 mM) on both sides from the beginning of the experiments. The histograms represent mean values ±S.E. (number of animals = 7). $^{\bullet}p < 0.05$; $^{\bullet\bullet}p < 0.01$ compared with the control period (10–40 min); paired data analysis. $^{*}p < 0.05$ compared with the same control period; unpaired data analysis.

statistical significance compared with the controls at any time during the experiment (fig. 5A, 5B).

Discussion

Effects on D-glucose transport. Experiments on intact tissue under short-circuit conditions confirm [7] that DAGO inhibits basal I_{sc} values. Once again, the decrease can be attributed to a variation in the transepithelial Na^+ and Cl^- net fluxes which are electrogenic and responsible for the short-circuit current [28]. The increase in Na^+, Cl^- and H_2O absorption produced by the addition of DAGO in open circuit conditions (everted sac technique) indicates a reduction in electrogenic Cl^- (and water) secretion, the consequence of which is a reduction in I_{sc} [7, 29]. The stimulatory effect of DAGO on $\Delta I_{sc,glu}$ is in accordance with the increase in D-glucose transport observed in the everted sac experiments. This confirms that enkephalins can modify the absorption of other nutrients apart from amino acids. It should be noted that in intact tissue the stimulatory effect on D-glucose transport is accompanied by a similarly oriented increase in ion and water transport, as if the two effects were linked. This may be due to the water streaming effect along the paracellular route. However, the experiments conducted on tissues deprived of muscularis and myenteric plexus demonstrate that the slow effect of DAGO on basal I_{sc} can still be observed, while there is no stimulating effect on D-glucose transport. This indicates that the two effects are not directly linked. In addition, it should be borne in mind that DAGO has opposite effects on D-glucose transport (stimulation) and L-valine transport (inhibition). Since both D-glucose and L-valine transepithelial transports are based on Na^+-associated cotransports on the apical side, it is unlikely that the same interference with basal ion and water transport can influence the two cotransports in opposite ways.

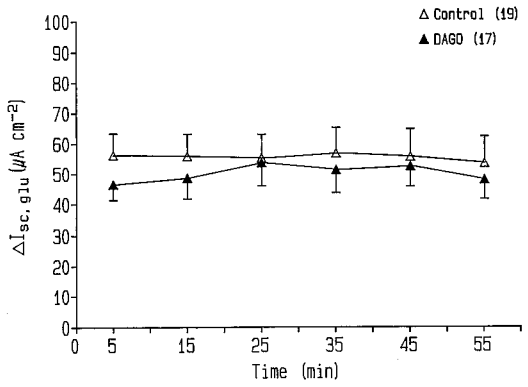

Figure 3. Stripped tissue: effects of 10^{-5} M DAGO on $\Delta I_{sc,glu}$. The data points represent mean value $\pm$S.E. (number of animals in brackets).

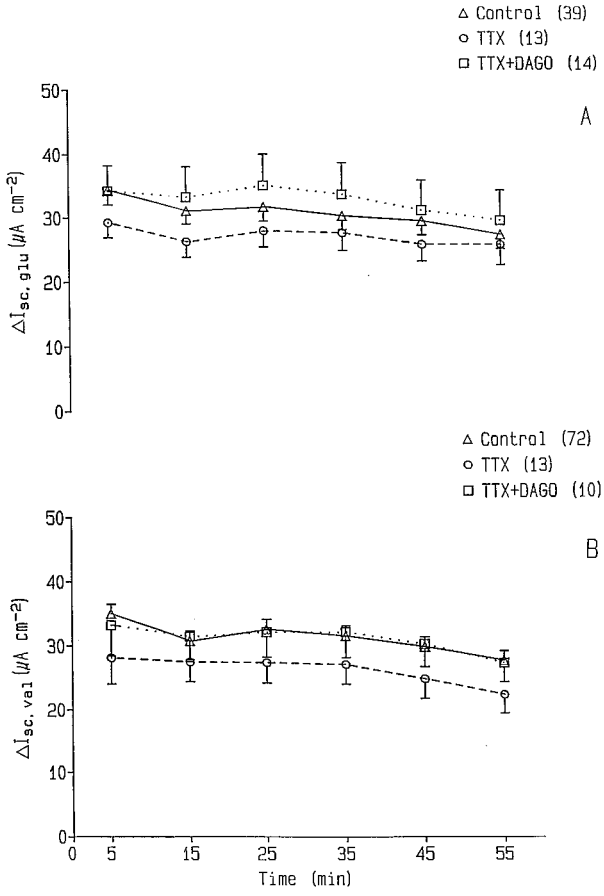

Figure 4. Intact tissue: effect of TTX (3×10^{-7} M) and TTX + DAGO (10^{-5} M) on $\Delta I_{sc,glu}$ (A) and L-valine dependent variations in I_{sc} ($\Delta I_{sc,val}$; B). The data points represent mean values $\pm$S.E. (number of animals in brackets).

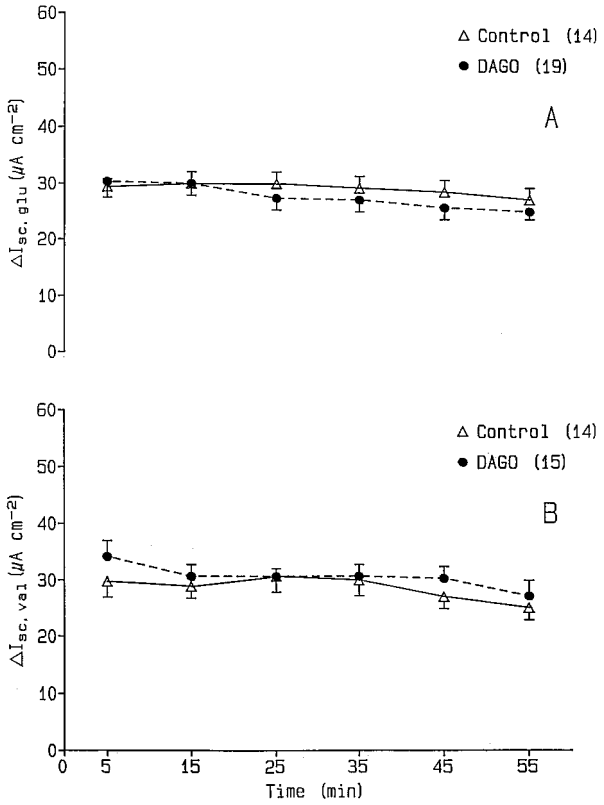

Figure 5. Intact tissue: effect of DAGO (10^{-5} M; mucosal side) on $\Delta I_{sc,glu}$ (A) and $\Delta I_{sc,val}$ (B). The data points represent mean values $\pm$S.E. (number of animals in brackets).

The apical increase in D-glucose transport could merely be a consequence of the rise in D-glucose metabolism caused by DAGO. In fact, experiments performed with 3-O-MG, which is not metabolized [27] but uses the same apical carrier as D-glucose (SGLT1: similar specificity for D-glucose and 3-O-MG [30]), show that the effects of DAGO on $\Delta I_{sc,3\text{-}O\text{-}MG}$ are not significantly different from its effects on $\Delta I_{sc,glu}$. Moreover, increased D-glucose metabolism could not in itself justify net increased transepithelial transport of D-glucose, as we observed in the everted sac experiments.

Mechanism of action of enkephalins. DAGO acts mainly on the μ-receptors, and its effects on D-glucose transport seem to be specific as they are antagonized by naloxone, as might be expected in such a case. Moreover, DADLE and the more specific (pCl-Phe^4)-DPDPE, which act on the δ-receptors, have no effect. This strongly suggests that the action of enkephalin on transport is mediated by the μ-receptors. The action must be mediated by the myenteric plexus, since DAGO has no effect on $\Delta I_{sc,glu}$ in its absence. The use of TTX helped to clarify this point. This substance, which blocks voltage-dependent Na^+ channels, has been used

in many studies to evaluate the nervous component in intestinal transport regulation [4]. It can inhibit the action of the nervous plexa, preventing the alterations induced by the release of endogenous neuromodulators. TTX alone does not modify nutrient transport. However, when added on the serosal side together with DAGO, it prevents the effects of enkephalin on $\Delta I_{sc,glu}$ and $\Delta I_{sc,val}$. This agrees with the fact that enkephalin receptors are located in the two enteric plexa (myenteric and submucosal). Most of the neurons of the submucosal plexus have projections on the intestinal epithelium, which suggests that the submucosal plexus is the only one involved in regulating the absorption and secretion processes. The myenteric plexus is believed to perform its action by controlling the smooth muscle layer. However, the ganglion cells of the two plexa are connected, indicating that they actually act as a single integrated unit [31, 32]. Our data suggest that the myenteric plexus plays a major part in D-glucose absorption. This agrees with the fact that opioid receptors have not been identified on the enterocytes [15, 16, 33]. In view of the effects on amino acid [7] and glucose transport, it can be concluded that enkephalins probably influence the release of an unidentified chemical neuromodulator from the enteric plexa. However, there is no clear indication of a regulatory effect on amino acid and D-glucose absorption by acetylcholine or noradrenaline, or by neuropeptides such as somatostatin and VIP [34, 35]. As regards the possible mechanism of action of enkephalins, involvement of the apical membrane receptors in the effects on nutrient absorption can be ruled out, since the addition of enkephalin on the mucosal side had no effect.

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