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Original article

Absorption of intact β-casomorphins (β-CM) in rabbit ileum *in vitro*

S. Mahé, D. Tomé, A-M. Dumontier and J-F. Desjeux

INSERM, Unité 290, fonctions intestinales, métabolisme et nutrition, hôpital Saint-Lazare, 107, rue du Faubourg St-Denis, 75010 Paris, France

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Summary — The functional significance of the presence of opioid peptides in enzymatic digestion of bovine milk β-casein remains unclear. Opiates modify intestinal electrolyte transport by acting on receptors located on the serosal side of the intestine. The aim of the present study is to determine under which conditions β-casomorphins could act from the luminal side of the intestine. The effect of natural morphiceptin (β-CM_4–NH_2) and the non metabolized analogue β-[$DAla^{2,4}$, Tyr^5]–CM_5–NH_2 were studied on isolated rabbit ileum mounted in Ussing chambers. Both peptides caused a naloxone-reversible reduction in short-circuit current (Isc) and stimulated Na and Cl absorption after addition to the serosal side of the tissue. After mucosal addition, only the analogue (10^{-3}M) crossed the epithelium intact (Jm–s=3.5 ± 1.2 nmol.h^{-1}.cm^{-2}) and reduced Isc. Morphiceptin, under the same conditions, was degraded by the intestinal mucosa without opiate action on electrolyte transport. Pretreatment of the ileum by 10^{-3}M diisopropylfluorophosphate that inhibited brush-border dipeptidylpeptidase IV, prevented mucosal degradation of morphiceptin. Under these conditions, the peptide (10^{-3}M) crossed the epithelium intact (Jm–s=1.8 ± 0.16 nmol.h^{-1}.cm^{-2}) and stimulated electrolyte absorption by means of an opioid mechanism. These results show that both natural morphiceptin and the protected analogue have an opiate activity on intestinal electrolyte transport. Their action from the lumen depends on their transfer intact to the serosal side of the intestine where opiate receptors are located. The limiting step in this transfer is at the brush-border membrane where dipeptidylpeptidase IV in particular seems to play a major role.

peptides — casein — opiates — intestin — ions transport

Résumé — Absorption de β-casomorphines (β-CM) sous forme intacte par l'iléon de lapin *in vitro*. *La signification physiologique de la libération des peptides opiacés (β-CM) au cours de la digestion luminale des β-caséines du lait n'est pas claire. Les opiacés modifient le transport intestinal des électrolytes en agissant sur des récepteurs localisés sur le versant séreux de l'intestin. Le propos de ce travail est de déterminer dans quelles conditions les β-casomorphines peuvent agir à partir du versant luminal de l'intestin. Les effets de la morphiceptine (β-CM_4–NH_2) et d'un analogue non métabolisable β–[$DAla^{2,4}$,Tyr^5]–CM_5–NH_2 sont étudiés* in vitro *sur des iléons de lapin montés en chambre de Ussing. Les deux peptides provoquent une réduction du courant de court-circuit (Isc) après addition dans le réservoir séreux. Cette réduction est inversée par la naloxone. Seul l'analogue peut traverser la muqueuse sous forme intacte (Jm–s = 3,5 ± 1,2 nmol.h^{-1}. cm^{-2}) après addition (10^{-3}M) côté muqueux et provoquer une réduction de Isc. Dans les mêmes conditions, la morphiceptine est hydrolysée par la muqueuse et n'a pas d'effet opiacé. Le traitement de la muqueuse par*

du diisopropylfluorophosphate (10^{-3}M) qui inhibe la dipeptidylpeptidase IV membranaire, protège la morphiceptine de l'hydrolyse. Le peptide (10^{-3}M) traverse alors la muqueuse sous forme intacte (Jm–s= 1,8 ± 0,16 nmol.h^{-1}.cm^{-2}) et stimule une absorption des électrolytes par un mécanisme opiacé. Ces résultats montrent que la morphiceptine et l'analogue non métabolisable β-[DAla^{2,4}, Tyr^5]–CM_5–NH_2 ont une activité opiacée sur les flux d'électrolytes. Cette action à partir du versant luminal de l'intestin dépend de leur transfert sous forme intacte vers le versant sanguin où les récepteurs opiacés sont localisés. L'étape limitante de ce transfert est localisée dans la bordure en brosse dans laquelle la dipeptidylpeptidase IV semble jouer un rôle clé.

peptide — caséine — opiacé — intestin — transport ionique

INTRODUCTION

Naturally occuring β-casomorphins (β-CM) are a family of food-derived opioid peptides obtained from the (60–66) Tyr-Pro-Phe-Pro-Gly-Pro-Ileu fragment of bovine β-casein (Brantl *et al.*, 1979; Henschen *et al.*, 1979; Lottspeich *et al.*, 1980). The tetrapeptide amide fragment (Tyr-Pro-Phe-Pro-NH_2), called morphiceptin, was also isolated from enzymatic digests of milk protein (Chang *et al.*, 1981, 1985). Finally, β-CM immunoreactive material was found in human intestine after ingestion of bovine milk (Svedberg *et al.*, 1985). However, it was not clear whether β-CM modify intestinal electrolyte transport in the same way as enkephalins and under which conditions these peptides could act from the luminal side of the intestine where natural sequences were found.

In the present study, the degradation and transepithelial passage of natural morphiceptin and of the non-metabolized analogue β-[DAla^{2,4},Tyr^5]–CM_5–NH_2, and the resulting effect on electrolyte transport, were evaluated *in vitro* in rabbit ileum mounted in a Ussing chamber. The effect of morphiceptin was measured in the presence and absence of diisopropylfluorophosphate (DFP) which is know to inhibit the activity of the membrane brush-border post-proline endopeptidase dipeptidylpeptidase IV (DPP IV). Results show that natural β-CM and the non-metabolized analogue β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 act on intestinal electrolyte transport by means of an opioid mechanism. Their action, from the luminal side, depends on their transfer intact to the serosal side of the intestine where opiate receptors are located.

MATERIALS AND METHODS

Substances : Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) and the analogue β-[DAla^{2,4},Tyr^5]–CM_5–NH_2, (Tyr-DAla-Phe-DAla-Tyr-NH_2), were kindly provided by V. Brantl, and DFP was purchased from Sigma.

Using chamber experiments

White male New Zealand rabbits weighing 2-3 kg were killed by intravenous pentobarbital sodium injection. Pieces of stripped ileum were mounted between the two halves of a Lucite chamber (exposed area = 3.14 cm^2) and were bathed on each side by oxygenated (95% O_2–5% CO_2) Ringer solution consisting of (in mM) 140 Na^+, 5.2 K^+, 120 Cl^-, 25 HCO_3^-, 1.2 Ca^{2+}, 2.4 HPO^{2-}_4, 0.4 $H_2PO_4^-$ and 1.2 Mg^{2+} (pH 7.4). The spontaneous transmucosal electrical potential difference (PD) was short-circuited by a short-circuit current (Isc) delivered by an automatic voltage clamp (WPI, USA) as previously described (Tomé *et al.*, 1987). The tissue conductance (G) was calculated according to Ohm's law. After checking the stability of electrical parameters, the peptide to be tested was added to the appropriate reservoir and aliquots were col-

lected from each reservoir at different times for HPLC analysis. Transepithelial unidirectional Na and Cl fluxes from mucosa to serosa (Jm–s) and from serosa to mucosa (Js–m) were measured using ^{22}Na (2 μCi) and ^{36}Cl (2.5 μCi), as previously described (Hautefeuille *et al.*, 1986). Whenever the effect of DFP was investigated, it was added to each reservoir to give the desired concentrations and the tissue was incubated 30 min to stabilize electrical parameters before assay.

Brush-border membrane vesicle experiments

Brush-border membrane vesicles (BBMV) were prepared from everted frozen rabbit ileum (Brot-Laroche & Alvarado, 1984; Schmitz *et al.*, 1973). Degradation of morphiceptin in the presence of a purified BBMV suspension was measured by HPLC, and DPPIV activity was evaluated according to Miyamoto *et al.* (1987).

High-pressure Liquid Chromatography (HPLC)

Peptides were analyzed with an HPLC system (Waters) equipped with an ultraviolet detector at 214 nm and a μ-Bondapack C18 reversed phase column (3 x 350 mm) eluted with linear 0–75% acetonitrile gradient (Tomé *et al.*, 1987).

RESULTS

Effects of β-CM on ion transport in rabbit ileum

Addition of either morphiceptin or β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 in the serosal

Table I. Effect of serosal addition of morphiceptin (β-CM_4-NH_2) and of the analogue β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 on ion transport in untreated rabbit ileum mounted in a Ussing chamber.

			JN_a			JCl	
	Isc	*m-s*	*s-m*	*net*	*m-s*	*s-m*	*net*
Control	1.06 ± 0.07	8.13 ± 1.37	9.24 ± 1.33	− 1.10 ± 0.28	7.24 ±0.92	7.36 ± 0.81	− 0.54 ± 0.48
β–CM_4-NH_2 10^{-3}M Serosal	0.73 ± 0.14	11.07 ± 1.09	10.53 ± 1.06	0.52 ± 0.15	8.81 ±0.76	7.27 ± 0.64	1.53 ± 0.56
P	< 0.05	NS	NS	< 0.05	NS	NS	< 0.05
Control	1.46 ± 0.14	6.71 ± 0.13	6.54 ± 0.62	0.17 ± 0.58	6.06 ± 0.56	6.60 ± 0.40	− 0.54 ± 0.54
β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 10^{-4}M Serosal	1.15 ± 0.15	7.97 ± 0.87	6.22 ± 0.69	1.76 ± 0.41	7.17 ± 0.64	5.99 ± 0.45	1.18 ± 0.53
P	< 0.05	NS	NS	< 0.05	NS	NS	< 0.05

Values are means ± SE of results for 6 to 10 animals. Isc and fluxes (J) are expressed in μeq.h^{-1}.cm^{-2}.m–s : unidirectional fluxes from mucosa to serosa, s–m : unidirectional fluxes from serosa to mucosa, net=ms–sm.

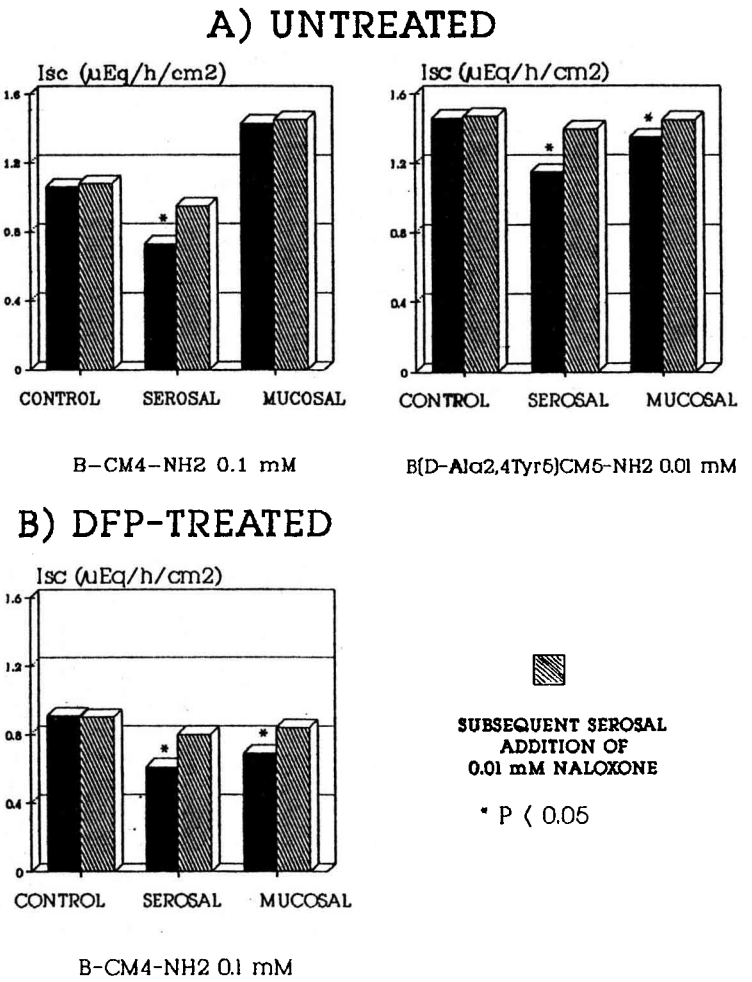

Fig. 1. Electrical response to addition of morphiceptin (β-CM_4–NH_2), β-[$DAla^{2,4}$,Tyr^5]–CM_5–NH_2 or no peptide for control, to serosal or mucosal side of untreated (A) and DFP-treated (B) rabbit ileum in a Ussing chamber. Isc values are means of results for 6 to 10 animals measured in the 15–60 min period after addition of the peptide (black area), and in the 15 min period after subsequent addition of naloxone (hatched area). SE values are in the 0.07–1.15 range. * : Isc is significantly reduced in comparison to the control ($P < 0.05$).

side of rabbit ileum produced a naloxone-reversible reduction in Isc, whereas, after mucosal addition, only the protected analogue β-[DAla^{2,4},Tyr^5]–CM$_5$–NH$_2$ produced the same naloxone-reversible effect on Isc (Fig. 1A). For both peptides, the effect on Isc was associated with a stimulation in net Na and Cl absorption (Table I). In DFP-treated tissue, both serosal and mucosal addition of morphiceptin produced a naloxone-reversible decrease on Isc (Fig. 1B).

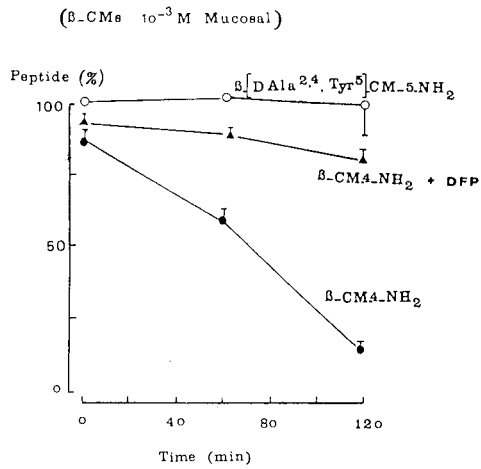

Fig. 2. Mucosal degradation of morphiceptin (β-CM$_4$–NH$_2$) in the presence or the absence of DFP and of β-[DAla^{2,4}, Tyr^5]–CM$_5$–NH$_2$ after addition in the mucosal side of rabbit ileum mounted in a Ussing chamber (exposed area : 3.14 cm^2). DFP was added 10^{-3} M to both serosal and mucosal reservoirs 30 min before morphiceptin was introduced.

Degradation and transepithelial passage of β-CM in rabbit ileum

In the absence of DFP, morphiceptin was rapidly degraded when introduced in the mucosal side of rabbit ileum *in vitro* and the protected analogue remained undegraded in the same conditions, whereas, after pretreatment of the tissue with 10^{-3}M DFP, the mucosal degradation of morphiceptin was prevented (Fig. 2). The content of the serosal reservoir was analyzed by HPLC after two hours of incubation of the peptides on the mucosal side of the tissue (Fig. 3). In the presence of untreated tissue, a well resolved peak was recovered in exactly the position of the protected analogue, whereas, intact morphiceptin could not be detected under the same conditions. On the other hand, morphiceptin was

Table II. Effect of increasing concentrations of DFP on the dipeptidylpeptidase IV (DPP IV) activity of brush-border membrane vesicles (BBMV) and their capacity to hydrolyse morphiceptin.

		DFP (M)		
	Control	*10^{-5}*	*10^{-4}*	*10^{-3}*
β-CM$_4$–NH$_2$ degradation (%)	100	60 ± 1.88	31 ± 1.19	08 ± 1.92
DPP IV activity (%)	100	90 ± 2.86	50 ± 2.23	10 ± 2.40

DPP IV activity and degradation of morphiceptin are expressed as a percent of the activity or of the degradation obtained with control untreated BBMV. BBMV were treated with increasing concentrations of DFP 30 min before 10^{-3}M morphiceptin was added to the suspension (n = 5).

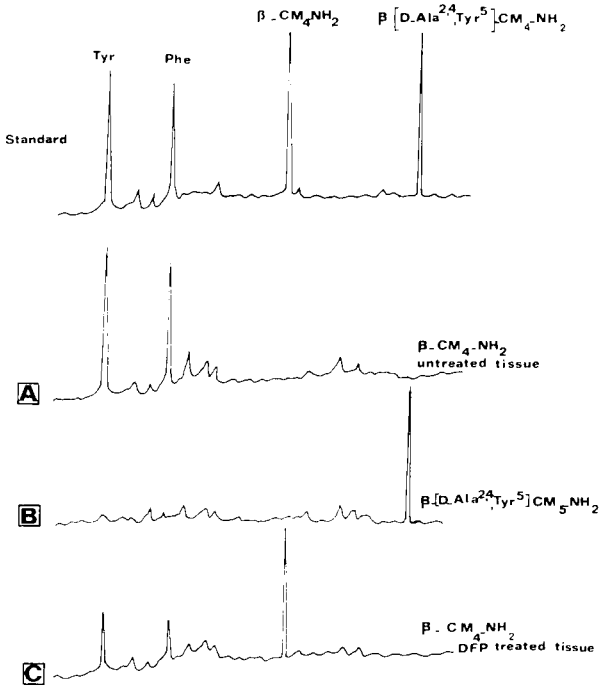

Fig. 3. Reversed-phase high-pressure liquid chromatography elution profile of serosal reservoir content after incubation for 120 min of (A) untreated tissue with 10^{-3} M morphiceptin (β-CM_4–NH_2) in the mucosal reservoir, (B) untreated tissue with 10^{-4}M β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 in the mucosal reservoir and (C) DFP-treated tissue with 10^{-3}M morphiceptin in the mucosal reservoir.

recovered in the serosal reservoir in the presence of the DFP-treated tissue.

Morphiceptin was also degraded after one hour of incubation in the presence of BBMV, but this degradation was inhibited by increasing concentrations of DFP in the 10^{-5}–10^{-3}M range with subsequent inhibition of DPP IV activity (Table II).

DISCUSSION

The present study confirms that β-CM affect intestinal ion transport in the same way as enkephalins since they reduced Isc by a mechanism reversed by naloxone and stimulated Na and Cl absorption simultaneously when added to the serosal side of isolated rabbit ileum (Dobbins *et al.*, 1980; Mc Kay *et al.*, 1981). In addition, it appeared that when peptides were added to the luminal side of the intestine, the expression of their opiate activity needs the transfer of an active form to the blood side of the tissue where their receptors are probably located (Tomé *et al.*, 1987). This finding is in accordance with other studies that demonstrated that opiate receptors are not present on the membrane of the enterocytes but are mainly located on submucosal and myenteric plexus (Dashwood

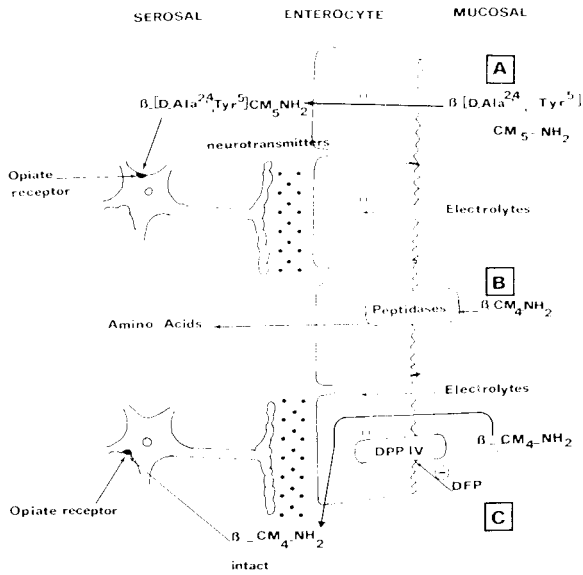

Fig. 4. Degradation and transepithelial transport of β-CM in the intestine. (A) The non metabolized analogue β-[DAla^{2,4},Tyr^5]–CM_5–NH_2 crosses the epithelium intact and acts on opiate receptors located on the serosal side; (B) Morphiceptin is hydrolyzed by brush-border peptidase DPP IV and has no opiate activity; (C) Inhibition of the DPP IV by treatment with DFP prevents mucosal degradation of morphiceptin (β-CM_4–NH_2) which crosses the epithelium intact and stimulates electrolyte absorption by means of an opioid mechanism.

et al., 1985; Gaginella *et al.*, 1983; Nishimura *et al.*, 1986; Tomé *et al.*, 1988).

Since β-CM are of alimentary origin, any natural physiological function of these peptides imply the existence of mechanisms that control their transfer across the intestinal mucosa to reach their targets. Our results demonstrate the existence of a system of degradation, probably located at the brush-border membrane and which effectively controls the absorption and the resulting opiate action of β-CM in the intestine (Fig. 4). This system represents a barrier for natural β-CM which are hydrolyzed and do not cross the intestine intact. However, utilisation of a nonmetabolized analogue demonstrated that, in untreated tissue, a peptide that escapes the system of degradation is able to cross the tissue intact; however, the mechanism by which it is transported it still not clear. In addition, natural morphiceptin was also able to be transferred intact across the ileum after treatment of the tissue by DFP. Though DFP is known to inhibit various enzymes, its protective effect on morphiceptin was correlated with a subsequent inhibition of the post-proline endopeptidase, DPP IV, which was already demonstrated to be very active in hydrolysing β-CM substrates (Hartrodt *et al.*, 1982; Kreil *et al.*, 1983; Miyamoto *et al.*, 1987). DPP IV is known to

be located in the brush-border membrane (Auricchio *et al.,* 1978). In those conditions, the limiting step in the transfer of intact β-CM across ileum seems to be located at the brush-border membrane, and DPP IV in particular, could play a major role in this barrier. Further studies are needed to determine the physiological conditions that modulate this system of degradation.

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