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β-Casomorphins alter the intestinal accumulation of L-leucine

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Everted sacs of the rat jejunum change the accumulation of [^{3}H]leucine when β-casomorphins (BCMs) or synthetic analogs, in a concentration range of 10^{-8} mol/l, are coincubated with the amino acid. BCM5 (BCM fragment 1–5, Tyr-Pro-Phe-Pro-Gly) and [D-Ala^2]-BCM5-NH$_2$ (Tyr-D-Ala-Phe-Pro-Gly) increase, whereas [L-Pro^4]-BCM5 (Tyr-Pro-Phe-D-Pro-Gly) decreases the leucine accumulation and [Arg^8]-vasopressin has no effect. No effect of BCM5 could be observed on the accumulation of the space marker [^{14}C]inulin. Specific binding sites for casomorphins were detected microautoradiographically, exclusively at the epithelial cell layer using [^{3}H][D-Pro^4]-BCM5 in competition studies as a model. HPLC analysis revealed that under the experimental conditions about 50% of the studied [D-Pro^4]-BCM5 was enzymatically degraded and no intact peptide is accumulated within the samples of everted sacs. From the results we postulate a brush-border receptor contact of the BCMs which induces an alteration of the amino acid uptake. A contraluminal binding of the chemical signals is not likely, because there is no evidence for a transepithelial transport of intact BCMs. The observed effects of the BCMs demonstrate as yet unknown peptide–receptor interactions, probably at the brush-border membrane, with subsequent effects on the nutrient supply. Furthermore, the results support the general hypothesis of distinct peptide–receptor interactions in those types of epithelia in which the cells are connected by tight junctions.

Introduction

β-Casomorphins (BCMs) are segments of the β-casein of the bovine milk [1] which preferentially bind to opioid receptors [2,3]. In the intestine, BCMs should originate by enzymatic cleavage of β-casein after milk intake [4]. Therefore, under physiological conditions these peptide molecules would contact the luminal surface of the intestinal epithelium.

It was our intention to investigate whether BCMs induce alterations of the transfer of leucine at the epithelium of the intestine. From previous studies with [Arg^8]-vasopressin, a blood borne nonapeptide, it is known that this peptide changes the blood-to-brain transfer of amino acids [5–7]. The blood-brain barrier as well as the intestine are both composed of cells which are connected by tight junctions. [Arg^8]-Vasopressin is bound to luminal receptors of the tight epithelium of the brain vessels and decreases V_{max} and K_m for amino acids [5,7,8]. This effect is ligand specific. BCM5, for instance, in contrast to [Arg^8]-vasopressin had no effect. Interestingly, BCMs are not obligatory components of the blood compartment [9].

There is evidence that ligands which bind to opioid receptors, e.g., β-endorphin, enkephalins, dynorphin (for review, see Ref. 10), BCMs [11,12] and enkephalin-like pentapeptide [13], induce alterations of the intestinal electrolyte transport. In such cases, the ligands occupy receptor sites at the basal surface of the intestinum. However, it was our intention to pay attention to the problem whether BCM molecules contacting the luminal surface of the intestinum induce alterations of the transport of nutritive compounds. An effect on the transfer of leucine at the epithelium of the intestine induced by BCMs would suggest two hypotheses: (1) BCMs are exogenous peptidic signals which induce transfer processes across biological membranes. (2) Distinct transfer processes across epithelia with tight junctions are regulated by peptides.

Abbreviations: BCM, β-casomorphin; BCM5, BCM fragment 1–5 (Tyr-Pro-Phe-Pro-Gly); [D-Ala^2]-BCM5-NH$_2$, Tyr-D-Ala-Phe-Pro-Gly-NH$_2$; [D-Pro^4]-BCM5, Tyr-Pro-Phe-D-Pro-Gly.

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Materials and Methods

Preparation of everted sacs

The jejunum of male Wistar rats (200–350 g), killed by cervical dislocation following a blow on the head, was removed and preincubated in a 37°C Krebs-Henseleit bicarbonate buffer solution (Na^+ 117, KCl 4.69, $CaCl_2$ 2.35, KH_2PO_4 1.17, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 24.6 mmol/l) plus glucose (5 mmol/l) bubbled with 95% O_2 and 5% CO_2

By turning the mucosal side out with the help of glass rods everted sacs of about 2 cm length of jejunum were quickly prepared and tied at both ends

Incubation of everted sacs

Single everted sacs of successive parts of the jejunum were transferred to vials containing the buffer solution (15 ml) with either L-[^{3}H]leucine or [^{14}C]inulin as tracers After incubation times of 15, 30, 45 or 60 s, respectively, and 3, 5, 7 and 10 min each sac was washed in an ice-cold buffer and cut quickly into a sample of about 25 mg After weighing, the samples were dissolved in Protosol and its radioactivity, as well as that of the incubation media, determined by liquid scintillation counting To test the effect of peptides, the following peptides were added to the vials containing the tracers and maintained at the indicated concentrations BCM5, $0.6 \cdot 10^{-8}$ mol/l, [D-Ala^2]-BCM5-NH$_2$, $0.6 \cdot 10^{-8}$ mol/l [D-Pro^4]-BCM5, $0.8 \cdot 10^{-8}$ mol/l, [Arg^8]-vasopressin, $1 \cdot 10^{-8}$ mol/l In the test series, we took care (1) that control vials, and those containing the peptide as well, were handled identically, and (2) the control and the experimental sac came from adjacent segments of the intestine

Calculation of the accumulation and the uptake of the tracers

The accumulation of the tracers in the everted sacs ($A_{(t)}$) was calculated from the equation

$$A_{(t)} = \frac{R_{int}\ 100}{R_{med}\ W_{int}} \quad (\%/g) \qquad (1)$$

where R_{int} represents the radioactivity of the everted sac sample, W_{int} its weight and R_{med} the radioactivity of the incubation medium per ml The uptake of L-[^{3}H]leucine (U_{Leu}) was calculated from the equation

$$U_{Leu} = \frac{(A_{Leu} - A_{in})}{100} \cdot C \ (\text{pmol/g}) \qquad (2)$$

where A_{Leu} and A_{in} represent the accumulation for [^{3}H]leucine or [^{14}C]inulin, which was used as a marker for extracellular space ('inulin space') C represents the concentration of L-leucine in mol/ml

Localization of casomorphins

Tyr-Pro-[^{3}H]Phe-D-Pro-Gly was used to localize the peptide by HPLC analysis and by microautoradiography For HPLC analysis the everted sacs were incubated in the buffer system containing additionally the radioactive peptide ($2.9 \cdot 10^{-8}$ mol/l) After different incubation periods the sacs were washed in buffer containing the nonlabeled analog in a concentration of $6.8 \cdot 10^{-6}$ mol/l Later, the sacs were weighed and homogenized and the radioactivity was estimated The HPLC analysis (HP 1084 B, United Packard) included both, everted sacs and the incubation fluid

For the microautoradiographic localization everted sacs, and, for comparison, another tissue of the animal, the diaphragma, were incubated for 10 or 30 min either with the tritiated analog ($4 \cdot 10^{-8}$ mol/l) alone or in an incubation medium containing both the labelled and the unlabelled peptide (about 2200-fold higher concentrated, $9 \cdot 10^{-5}$ mol/l), for competition Later, the washed and frozen (−180°C, Isopentan) tissue samples were cut in a Cryostat to 12-μm sections which were thaw mounted [14] on a photographic emulsion (ORWO K 6) After a 200 days exposure at −15°C the photographically processed autoradiographs were stained with Toluidine blue For the evaluation of the autoradiograms silver grains were counted over an area of 4000 to 15000 μm^2 of the following locations: (1) the inner space of the everted sacs, (2) the muscle layer of the jejunum, (3) the epithelial layer, (4) the mucus surrounding the epithelium, and (5) the muscle tissue composing the diaphragma

Statistics

Data were evaluated statistically by the matched-pairs two tailed WILCOXON-test when the sacs were obtained from adjacent intestinal segments The U-test by Mann and Whitney was used for evaluating the autoradiographic studies Nonlinear regression estimations were obtained by unweighted least squares to calculate the theoretical curves

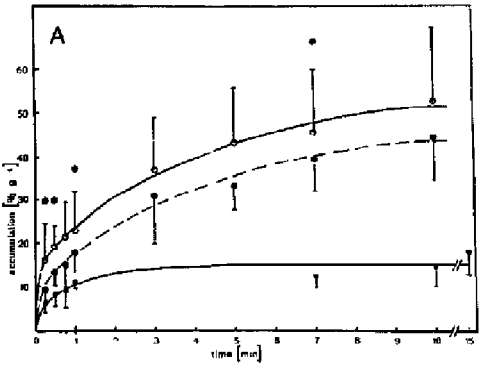
Materials

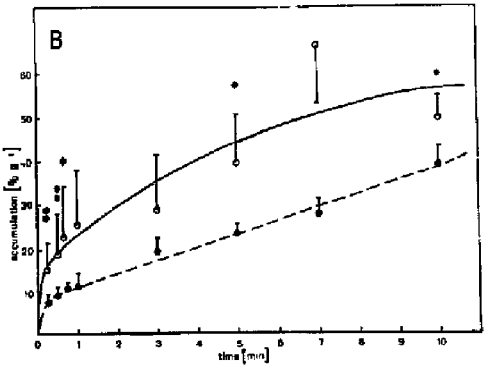
L-[4,5-^{3}H]Leucine (1.47–4.74 TBq/mmol) AVVR, Prague and Amersham International, [^{14}C]inulin (185–370 MBq/mmol) Amersham International, [Arg^8]-vasopressin Institute of Organic Chemistry and Biochemistry, Prague, BCMs (including [*Phe*-^{3}H][D-Pro^4]-BCM5, 695 GBq/mmol). Institute of Biochemistry, Halle, G.D.R., Protosol, New England Nuclear; POPOP and PPO Riedel DeHaen

Results

Accumulation of tracers and uptake of L-leucine

Everted sacs accumulate L-leucine and inulin depending on the incubation time (Fig 1A) The curve of the





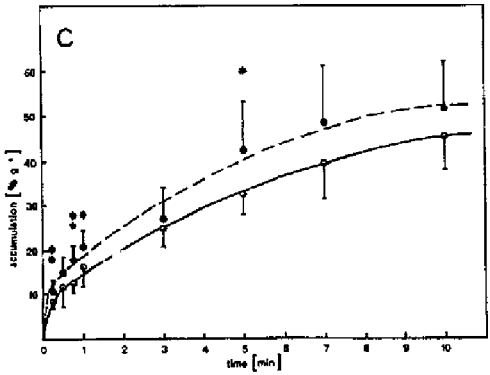


Fig. 1 Time-dependent accumulation of L-[^{3}H]leucine (●) or [^{14}C]inulin (■) in everted sacs ○, L-[^{3}H]leucine plus (A) BCM5 (0.6 10^{-8} mol/l), or plus (B) [D-Ala^2]-BCM5-NH$_2$ (0.6 10^{-8} mol/l), or plus (C) [D-Pro^4]-BCM5 (0.8·10^{-8} mol/l) Data are means ± S.D., n = 4–15 (A), 4–13 (B), 7–25 (C), respectively * $P \leq 0.05$, and ** $P \leq 0.01$ ● versus ○

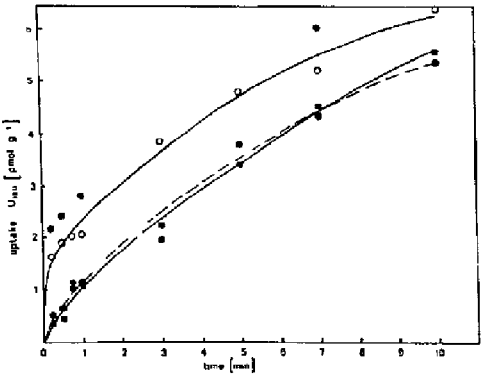


Fig. 2 Time dependent uptake of L-[^{3}H]leucine (●) in everted sacs. ○ L-[^{3}H]leucine plus BCM5 (0.6 10^{-8} mol/l), ■, L-[^{3}H]leucine plus [Arg^8]-vasopressin (10^{-8} mol/l) Data are means (n = 4–31) S.D. = 4 to 50% * $P \leq 0.05$ ○ versus ●

inulin-accumulation reaches a plateau after a few minutes, that of L-leucine after about 10 min A_{Leu} exceeds considerably the corresponding values of A_{in}, in the range of the plateau by a factor of about 2.8 Concubation of L-leucine with BCM5 (Fig. 1A), [D-Ala^2]-BCM5-NH$_2$ (Fig 1B) or [D-Pro^4]-BCM5 (Fig. 1C) but not [Arg^8]-vasopressin (Fig 2) alters the accumulation of L-leucine A_{in} remains unaltered after concubation of the space marker with BCM5.

The changes of A_{Leu} induced by the peptides are different BCM5 and [D-Ala^2]-BCM5 induce an increased, [D-Pro^4]-BCM5 a decreased accumulation of leucine The increase induced by both peptides is notable in all corresponding data, most significantly comparing corresponding values (Figs 1A and 1B) and amounts to as much as 80% (BCM5) or even 100% ([D-Ala^2]-BCM5-NH$_2$) [D-Pro^4]-BCM5 induced a decrease of A_{Leu} by 8 to 30% (Fig 1C).

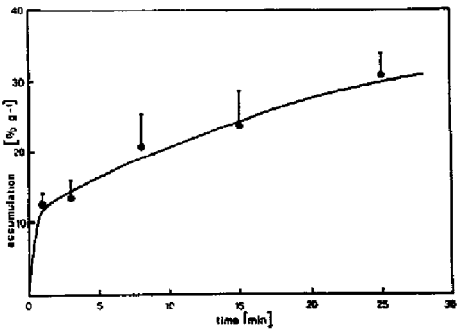


Fig. 3 Time-dependent accumulation of ^{3}H radioactivity in everted sacs incubated in a medium containing [^{3}H][D-Pro^4]-BCM5 (3 10^{-8} mol/l) Data are means (n = 5) ± S.D.

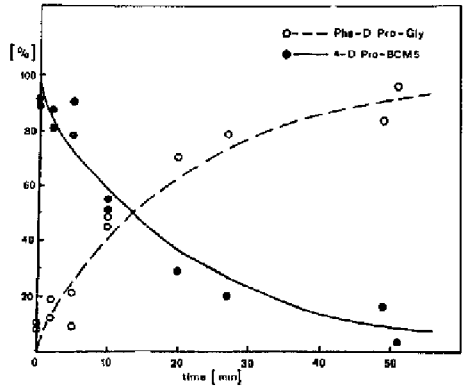


Fig 4 HPLC analysis of time dependent concentrations of [^{3}H][D-Pro^4]-BCM5 and its metabolite [^{3}H]Phe-D-Pro-Gly in the medium during the incubation of everted sacs ($n = 10$) at 37 °C [^{3}H][D-Pro^4]-BCM5 (3 10^{-8} mol/l) was added to the medium (15 ml) prior to the incubation of everted sacs Lines were drawn by computerized optimization

Converting A_{Leu} to U_{Leu} reveals that BCM5 actually significantly increases the transport of L-leucine into the tissues of the everted sacs, in contrast to [Arg^8]-vasopressin (Fig 2)

Localization of Tyr-Pro-[^{3}H]Phe-D-Pro-Gly

Fig 3 demonstrates the accumulation of radioactivity in everted sacs after the incubation with [^{3}H][D-Pro^4]-BCM5 The HPLC analysis revealed that the measured accumulation of radioactivity originated from the labeled phenylalanine in the tripeptide Phe-D-Pro-Gly and not from the intact pentapeptide The intact peptide

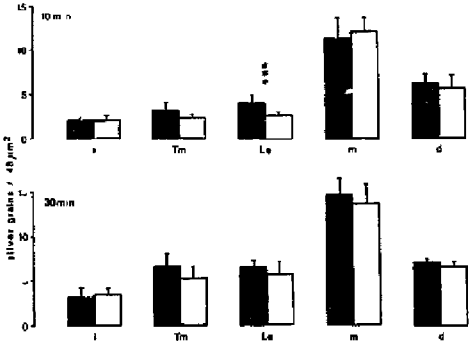


Fig 5 Number of silver grains (means ± S D) over regions of the rat jejunum and the diaphragm after the incubation of everted sacs for 10 or 30 min in the incubation medium containing [^{3}H][D-Pro^4]-BCM5 (4 10^{-8} mol/l) (black columns) or, for competition the labelled peptide together with the unlabelled (9 10^{-5} mol/l) (white columns) i, inner space of the sacs, Tm, muscle layers, Le, mucosal epithelium, m, mucus, d, diaphragma *** $P \leq 0.001$

could be demonstrated in the incubation medium (Fig 4) Calculations of the half-life from a sample containing ten everted sacs leads to a value of approx. 10 min

Autoradiographically, radioactivity was visualized over all locations studied (Fig 5) with the heaviest concentration in the mucus layer Under competition conditions only the labelling of the epithelial layer of the everted sacs was significantly diminished after the 10 min incubation with [^{3}H][D-Pro^4]-BCM5

Discussion

Everted sacs are an accepted model for studying transport processes of the mammalian intestine [15–20] Under the circumstances described, the accumulation of L-leucine (A_{Leu}) is a time-dependent and saturable process reaching a plateau in about 10 min The real uptake of L-leucine (U_{Leu}), 10^{-8} mol/l concentration was about 5 pmol/g per 15 min. This value corresponds with measurements of amino acid transport into isolated segments [21], rings [22] and everted sacs of rat small intestine [23]

Concubation of labelled L-leucine with peptides had different effects on the accumulation of the amino acid BCM5 and [D-Ala^2]-BCM5-NH$_2$ increased A_{Leu}, whereas [D-Pro^4]-BCM5 decreased and [Arg^8]-vasopressin had no effect Furthermore, no effect of BCM5 on the accumulation of inulin was observed Therefore, the phenomenon observed is signal (peptide) and substrate specific

Two problems arise from the results obtained (1) what might be the events underlying the effects observed? and, (2) what might be its significance?

Regarding the first problem, the data suggest an alteration of the carrier-mediated leucine transport at the brush-border membrane induced by a ligand–receptor interaction This explanation is underlined by the following arguments The BCMs alter only the saturable, that means the carrier mediated [16,24] component of the leucine transport There is no evidence of any alteration of the passive diffusion of the amino acid because the accumulation of the space marker inulin was not changed A BCM–receptor interaction at the epithelial layer of the everted sacs is postulated as a result of the microautoradiographic data (Fig. 5) Binding of [^{3}H][D-Pro^4]BCM5 was significantly inhibited by an excess of unlabelled peptide only at the epithelial layer The resolution power of the microautoradiographic technique used does not make it possible to decide whether the specific binding sites are localized luminal and/or abluminal at the epithelial layer But, two further groups of arguments indicate the brush border membrane as the primary site of the specific binding the origin of BCMs in vivo and the properties of the tight intestinal epithelium in regard to transport of peptides across it

In vivo, BCMs originate in the immediate neighbourhood of the brush-border membrane [1,4] Contact between the two, the peptide signal and the assumed specific membranous binding sites should occur as long as degrading enzymes [25] do not cleave the peptide molecules prior the interaction In the in vitro experiments presented (Fig 4), the half-life of [D-Pro^4]-BCM5, concentration 10^{-8} mol/l, amounts to about 10 min That concentration, which is on the lower limit of BCMs within the human gut after intake of 1 l bovine milk (10^{-8}–10^{-5}) [4], is sufficient to establish contact between the peptidic signal and the assumed receptor at the brush-border membrane within the time interval used Our results indicated that it was highly unlikely that the intact pentapeptide crosses the intestinal epithelium in significant amounts This conclusion is based on HPLC analysis, which revealed no intact peptide within samples of everted sacs (Fig 4) Furthermore the tightness of the epithelium for molecules like peptides or inulin is high considering the measured accumulation (Fig 1A) In addition, BCMs do not increase the inulin-space, that means they do not produce pathways for their penetration Tome et al [11] detected no transepithelial BCM5 passage in the rabbit ileum, whereas a BCM-analog, stable against enzymatic degradation, passes through up to 0 01 percent Taking into account these data, in our own experiments we can expect only a minute transepithelial transport of enzymatically resistent sequences of the stable analogs, leading to concentrations of about 10^{-11} mol/l in the serosal compartment An effect of such minute concentrations at opiate receptors within the muscle layer, e g. at the myenteric plexus, is questionable Brantl et al [2] detected that concentrations in the range of 10^{-6} mol/l of stable BCM-analogs are necessary for a 50% inhibition of the electrically induced contractions of the guinea pig ileum preparations

Changes of the intestinal amino acid transport, on the other hand, were found to be elicited by intravenously administered peptides (somatostatin [26], VIP, somatostatin, CCK [27]) or in isolated enterocytes (somatostatin [28]) In these cases, at least after the intravenous administration of the peptides, contact of the signals with the basolateral membranes of the epithelium should occur Therefore, although it would be very unlikely regarding the studies presented, an alteration of the intestinal amino acid transport induced by a basolateral contact of BCMs cannot be until now, totally excluded

Interestingly, a luminal peptide-receptor interaction assumed here for the intestinal epithelium seems to be a more general phenomenon. At the luminal site of the epi-(endo-)thelium representing the blood-brain barrier, [Arg^8]-vasopressin and some analogs, but not BCM5, induce carrier-mediated transepithelial amino acid transport alterations [5–7,29]. Furthermore, at the blood-brain barrier vasopressin-receptors have been detected [5,8,30]

The events underlying the different alterations of the amino acid transport induced by the different BCMs are not fully understood The higher accumulation induced by [D-Ala^2]-BCM5-NH_2 in comparison to BCM5 itself could possibly, be explained by its higher affinity to the assumed receptor Brantl et al [2] observed a higher affinity of the analog to δ-opiate receptors The lower leucine accumulation induced by the D-Pro analog remains unclear until real ligand receptor interactions have been measured

Regarding the significance of the effects observed we assume a support of the nutrient supply [31]

In conclusion (1) BCMs, originating by proteolytic degradation within the intestinal lumen come in contact with assumed receptors at the brush-border membrane of absorptive enterocytes (2) The peptide–receptor interaction induces alterations of the amino acid transfer across the intestinal epithelium. (3) This, as yet unknown, peptide–receptor interaction at the brush-border membrane with its probable effects on the nutrient supply support the general hypothesis of peptide–receptor interactions of those types of epithelia in which the cells are connected by tight junctions

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