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## $\beta$ -Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a $\mu$ -opioid pathway

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**Zoghbi, Sandra, Aurélien Trompette, Jean Claustre, Mahmoud El Homsy, Javier Garzón, Gérard Jourdan, Jean-Yves Scoazec, and Pascale Plaisancié.**  $\beta$ -Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a  $\mu$ -opioid pathway. *Am J Physiol Gastrointest Liver Physiol* 290: G1105–G1113, 2006. First published December 15, 2005; doi:10.1152/ajpgi.00455.2005.—We have recently shown that  $\beta$ -casomorphin-7, a milk opioid peptide, strongly stimulates mucin secretion in the rat jejunum through a nervous pathway and opioid receptor activation. In this study, the hypothesis that  $\beta$ -casomorphin-7 may also act directly on intestinal goblet cells was investigated in vitro in rat and human intestinal mucin-producing cells (DHE and HT29-MTX) using quantitative and semiquantitative RT-PCR and ELISA. The presence of  $\mu$ -opioid receptors was demonstrated in rat goblet cells in the upper half of the colonic crypt and in the two cell lines by immunohistochemistry and RT-PCR. In rat DHE cells,  $\beta$ -casomorphin-7 increased the expression of rat mucin (rMuc)2 and rMuc3 but not rMuc1, rMuc4, and rMuc5AC. This effect was time and dose dependent, with the maximum of increase in transcripts being noticed for a concentration of  $10^{-4}$  M after 2 h of stimulation for rMuc2 (225% of controls) and 4 h of stimulation for rMuc3 (208% of controls). Mucin secretion was maximally increased after 8 h of stimulation. Interestingly, these effects were prevented by pretreatment of the cells with the  $\mu$ -opioid antagonist cyprodime. In human HT29-MTX cells,  $\beta$ -casomorphin-7 ( $10^{-4}$  M) also increased MUC5AC mRNA levels (219% after 24 h of stimulation) and the secretion of this mucin (169% of controls). In conclusion,  $\beta$ -casomorphin-7 may contribute significantly to mucin production via a direct effect on intestinal goblet cells and the activation of  $\mu$ -opioid receptors. Because intestinal mucins have a crucial mucosal protective function, dairy products containing  $\beta$ -casomorphin-7 may improve intestinal protection and could have dietary and health applications.

rat mucin 2; rat mucin 3; mucin 5AC; mucus; milk bioactive peptides

THE GASTROINTESTINAL MUCUS GEL covering the mucosal surface is a major component of physiological defense mechanisms. Mucus separates mucosal cells from the exterior milieu, provides protection from noxious substances (e.g., acidity, proteolytic enzyme activities, or toxins), and constitutes a local physical barrier against bacteria and pathogens (11). Mucus also regulates epithelial hydration, allows lubrication of the cell surface, and participates indirectly in the immune response due to interactions with secretory immunoglobulins (15). Gastrointestinal mucus owes its properties to secretory mucins of the mucin (MUC) family. Four members of this family are generally thought to be able to form mucus gels: MUC2,

MUC5AC, MUC5B, and MUC6 (12). These mucins have distinct expression patterns along the human gastrointestinal tract. Normal stomach mucosa is characterized by the production of MUC5AC, primarily by surface epithelial mucus cells, and of MUC6 by the gastric glands. The epithelium of the small and large intestine contains characteristic goblet cells that produce MUC2. This mucin is the predominant secretory mucin in the healthy intestine of the human, rat, and mouse (4, 47, 48). In recent years, a second class of mucins, membrane-associated mucins, has received increasing attention for its role in the protection of epithelia (5). In the intestine, prominent membrane-associated mucins are MUC1, MUC3, and MUC4 (25). They provide a steric barrier that can limit direct access of pathogens.

Because mucins are strategically positioned between the vulnerable mucosa and the luminal contents of the bowel, any quantitative or qualitative modification of their secretion and/or expression may affect the efficiency of the protective barrier and may have important physiological or pathological implications. Many studies thus support the hypothesis that alterations in mucin synthesis, secretion, and/or degradation may be involved in the initiation or maintenance of intestinal diseases (13). For example, goblet cells are reduced in number and contain less mucin in active ulcerative colitis, thus inducing a loss of the mucus layer (13, 50). In this context, the strengthening of the mucus gel, in particular by nutrients, could be extremely beneficial (19). However, little is known about the potential effects of nutritional factors on intestinal goblet cells as well as on their mechanisms of action. Previous experiments carried out in vivo or in vitro have only shown that dietary fibers and short-chain fatty acids can modify the dynamics of mucus by increasing the secretion or expression of mucins or even the number of goblet cells (2, 18, 31, 39–41, 51). We also showed recently that a family of milk opioid peptides, the  $\beta$ -casomorphins ( $\beta$ -CM), induced a strong release of mucin in the jejunum of the rat through the activation of the enteric nervous system and opioid receptors (10, 46). In some other aspect, the presence of opioid receptors on intestinal cells suggests the possibility that  $\beta$ -CMs, which are produced in the intestinal lumen, might also control the production of mucin via a direct action on epithelial goblet cells. The present study was thus undertaken to evaluate the direct effect of  $\beta$ -CMs on the function of intestinal goblet cells. For this purpose, we used rat and human intestinal mucus-secreting cell lines as a model

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to avoid interactions with the nervous system. These cell lines, which are derived from the rat and human intestine, exhibit the characteristics of mucin-producing cells and provide reliable tools for the study of regulation of gastrointestinal mucin expression and secretion (27, 45).

## MATERIALS AND METHODS

### Materials

Media and reagents (DMEM, penicillin-streptomycin, and trypsin), TRIzol, RT-PCR reagents and enzymes, distilled RNase-free water, and the Random Primers DNA Labeling System were obtained from Invitrogen (Cergy Pontoise, France). The FastStart DNA Master SYBR Green I kit was from Roche diagnostics (Meylan, France). The biotinylated wheat germ agglutinin, biotinylated goat anti-rabbit antibody, and avidin/biotinylated peroxidase complex (Vectastain Elite ABC reagent) were provided by Vector laboratories (Burlingame, CA). Microtiter plates (NUNC-Immunoplate) were obtained from Polylabo (Strasbourg, France).  $\beta$ -CM-7 and (D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,glycinol<sup>5</sup>)enkephalin (DAMGO) were obtained from Bachem (Bubendorf, Switzerland). Other reagents were provided by Sigma (Saint Louis, MO).

### Cell Culture

The DHE cell line, a previously described mucin-producing rat colon adenocarcinoma cell line (45), was a generous gift of F. Martin (INSERM U517, Dijon, France). HT29-MTX, a human colon carcinoma-derived mucin-secreting goblet cell line, was provided by Dr. T. Lesuffleur (INSERM U560, Lille, France) (28). The two cell lines were grown in plastic 25-cm<sup>2</sup> culture flasks in DMEM supplemented with 10% FBS and 100 mg/ml penicillin or streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere in a humidified incubator.

To study the effect of  $\beta$ -CM-7 and DAMGO, cells were seeded in 12-well culture plates. Experiments were performed 3 (DHE cells) or 21 days (HT29-MTX cells) after confluency. Twenty-four hours before the studies, the culture medium was replaced by serum-free medium to starve the cells from serum and to eliminate any interfer-

ence from extraneous proteins or hormones. The experimental protocol was then the following: the serum-free medium was removed, and the monolayer cultures of DHE or HT29-MTX were washed twice with PBS (37°C). Serum-free medium with or without  $\beta$ -CM-7 or DAMGO was added to the cells and incubated at 37°C for 30 min to 24 h in a humidified atmosphere.  $\mu$ -Opioid receptor blockade was performed by preincubating the cells with cyprodime for 30 min before agonist addition. The supernatants were then collected, frozen, and stored at -20°C. Cells were processed with trypsin. The cell numbers per well were determined, and total RNA was isolated. All experiments were performed at least three times in triplicate.

### RT-PCR of Mucins, $\mu$ -Opioid Receptor, and Cyclophilin

Briefly, total RNA was extracted from the rat colon or DHE or HT29-MTX cells with TRIzol and was reverse transcribed as previously described (45). Mucins and  $\mu$ -opioid receptor cDNAs were amplified by PCR with primer sequences previously published (Table 1). Cyclophilin was amplified as a reference gene.

PCR was performed under the thermocycling conditions as follows: 2-min initial denaturation at 94°C, 30-s denaturation at 94°C, 1-min annealing at 60°C, and 1-min extension at 72°C. The last amplification was followed by a final 10-min elongation step at 72°C. The number of cycles was chosen to fall into the exponential phase of amplification. The identity of PCR products was confirmed by sequencing the amplicons (BIOFIDAL, Vaulx en Velin, France).

For semiquantitative analysis of mucin mRNA expression, PCR gels (2% agarose stained with ethidium bromide) were visualized and pixelized with the "Image System" (Quantum Appligene, Pleasanton, CA) and densitometrically analyzed with Scion image version 4.0.2.

### Real-Time PCR

Real-time PCR measures were performed with the real-time fluorescence detection method (Roche Diagnostics) using the LightCycler System. Primer sequences were designed with the assistance of computer software Primer3 (Table 1). The reaction mixture contained MgCl<sub>2</sub> (80 nmol), forward and reverse primers (8.12 nmol), and 2  $\mu$ l LightCycler Fast Start DNA Master SYBR Green I Mix in a volume

Table 1. Primers for semiquantitative and real-time PCR

Genes	Base Pairs	Primers	References
rMuc1	286	5'-TCGACAGGCAATGGCAGTAG-3' (154-173) 5'-TCTGAGAGCCACCACTACCC-3' (439-420)	43
rMuc2	589	5'-GCCTCAAACCGTGCGTGTGTC-3' (362-381) 5'-TCATTACCAACCACTCATC-3' (950-931)	29
rMuc2 for quantitative RT-PCR	245	5'-ATTACCCACAGTGGACAA-3' (517-536) 5'-GGGATGTCCACCACAAAGTT-3' (761-742)	Designed with Primer3 for T <sub>M</sub> = 60°C
rMuc3	335	5'-TCATCCTGAAGGCCAGTAC-3' (398-417) 5'-CTGACATTTGCCATAGCTGC-3' (732-713)	45
rMuc4	638	5'-CGTACTAGAGAACTGGACATGC-3' (4761-4783) 5'-GGTAGGAGAACTTGTCATGG-3' (5398-5378)	52
rMuc5AC	470	5'-TATGAGGTGCGACTGCTTTG-3' (726-745) 5'-CACTGGCGTGGGCTCAAAGA-3' (1195-1176)	24
Rat cyclophilin	180	5'-CTTGTCATGGCAAATGCTG-3' (326-345) 5'-GTGATCTTCTTGCTGGTCTTG-3' (505-485)	9
MUC5AC	409	5'-TGATCATCCAGCAGCAGGCT-3' (2897-2917) 5'-CCGAGCTCAGAGGACATATGGG-3' (3305-3284)	20
MUC5AC for quantitative RT-PCR	240	5'-CGACCTGTGTGTGTACCAT-3' (2870-2889) 5'-CCACCTCGGTGTAGCTGAA-3' (3109-3091)	Designed with Primer3 for T <sub>M</sub> = 60°C
Human cyclophilin	166	5'-TCCTAAAGCATACGGTCTGGCAT-3' (280-304) 5'-CGCTCCATGGCTCCACAATATTCA-3' (445-421)	8
Rat $\mu$ -opioid receptor	569	5'-ACCTGGCTCTGGCTCAACTT-3' (284-304) 5'-TGGACCCCTGCCTGTATTTTG-3' (852-832)	3
Human $\mu$ -opioid receptor	554	5'-AAGTCTCGGTGCTCCTGGCTAC-3' (184-205) 5'-GATCAACACTCATGGTCAGAGG-3' (737-715)	7

rMuc, rat mucin; MUC, mucin; T<sub>M</sub>, melting temperature.

of 10  $\mu$ l. The reaction mixture was distributed into precooled capillaries and diluted (1:10) cDNAs or purified, and quantified cloned plasmid DNA for mucin (standard curve) in a volume of 10  $\mu$ l was added as PCR template. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 10 s, a touchdown (0.5°C/cycle) annealing from 68°C to 60°C for 8 s, and elongation at 72°C for 6 s. Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase, and melting curves were acquired at the end of the run.

#### Immunohistochemical Procedures

Cells (25,000 cells/well) were cultured in eight-well chamber slides (Costar, Cambridge, MA). They were then fixed with 4% neutral buffered formaldehyde for 10 min at room temperature and rinsed with PBS. Cells were then incubated with rabbit polyclonal primary antibody against human or rat  $\mu$ -opioid receptor (1:200) (17) for 1 h. The slides were then rinsed five times with PBS and exposed to rhodamine-coupled goat anti-rabbit secondary antibody (1:50 in PBS) for 30 min in the dark. The slides were then rinsed, cleared, and mounted.

For demonstration of  $\mu$ -opioid receptors in rat colonic mucosa, paraffin-embedded sections were deparaffinized in methylcyclohexane and rehydrated through graded alcohols at room temperature. Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6.0) by microwave treatment. The sections were treated for 30 min in blocking solution (with 2% BSA and 10% fetal bovine serum in PBS) and then incubated for 60 min at room temperature with rabbit anti- $\mu$ -opioid receptor antibody (17) followed by anti-rabbit rhodamine-conjugated secondary antibody (1:100).

To determine whether  $\mu$ -opioid receptors were localized in mucin-producing cells, we used double fluorescence immunohistochemistry. The sections were incubated 60 min at room temperature with rabbit anti-mucin2 (H-300, 1:250, Santa Cruz Biotechnology, Santa Cruz, CA) antibody and then with anti-rabbit FITC-conjugated secondary antibody (1:100). The slides were rinsed five times with PBS and incubated with rabbit anti- $\mu$ -opioid receptor antibody (MOR-1; 1:100, Santa Cruz Biotechnology). The second immune complex was revealed with anti-rabbit rhodamine-conjugated antibody (1:100). The slides were then rinsed, cleared, and mounted.

#### Enzyme-Linked Lectin Assay

An enzyme-linked lectin assay (ELLA) was used to measure mucinlike glycoprotein secretion as previously described (45). Briefly, wells of a microtiter plate were coated with sample diluted in sodium carbonate buffer (0.5 M, pH 9.6) and incubated overnight at 4°C. The plates were then washed with PBS containing 0.1% Tween (PBS-Tween, pH 7) and blocked with 2% BSA in PBS-Tween for 1 h at 37°C. After samples were washed five times, biotinylated wheat germ agglutinin in PBS-Tween-BSA was added, and the samples were incubated for 1 h at 37°C. Colorimetric determinations using avidin-peroxidase conjugate and *o*-phenylenediamine dihydrochloride solution were performed at 492 nm.

Mucinlike glycoprotein content of samples was determined from standard curves prepared from DHE or HT29-MTX mucins isolated from 75-cm<sup>2</sup> flasks and purified by ultracentrifugation as described previously (45). The amount of mucinlike glycoprotein secreted in the incubation medium was expressed as nanograms of mucinlike glycoprotein per 10<sup>6</sup> cells, and the results were given as percentages of controls.

#### ELISA for Human and Rat Mucins

The secretion of rat (r)Muc2 by the DHE cells and of MUC5AC by the HT29-MTX cells was measured by an ELISA using the H-300 and 45M1 primary monoclonal antibodies (Santa Cruz Biotechnology), respectively, as previously described (45). Samples of incubation

medium were incubated for 24 h at 37°C in a 96-well plate. Plates were then washed three times with PBS containing 0.1% Tween and blocked with 2% BSA in PBS-Tween for 1 h. They were then washed again and incubated with 50  $\mu$ l of the mouse monoclonal antibody (1:100) for 1 h. The wells were then incubated with 100  $\mu$ l of biotinylated goat anti-mouse IgG conjugate (1:10,000) for 1 h. After three washes, 100  $\mu$ l of avidin-peroxydase conjugate were added, and plates were processed as described for the ELLA. Porcine gastric mucin, previously shown to react strongly with anti-human gastric mucin monoclonal 45M1 antibody (23), or purified rat intestinal mucin was treated in the same way to obtain a mucin standard curve. The results were given as percentages of controls.

#### Statistical Analysis

Data were compared using repeated-measures ANOVA, followed by the Mann-Whitney *U*-test when appropriate or Mann-Whitney test alone for single comparisons. Differences with  $P < 0.05$  were considered significant. Data were analyzed by using Statview 4.57 for Windows (Abacus Concept, Berkeley, CA) and are presented as mean  $\pm$  SE.

## RESULTS

#### *Rat Colonic Goblet Cells and DHE Cells Express the $\mu$ -Opioid Receptor*

$\beta$ -CMs are a family of  $\mu$ -selective opioid peptides. In this study, we investigated the hypothesis that  $\beta$ -CMs may act directly on goblet cells of the intestinal tract. We first determined the localization of  $\mu$ -opioid receptors at the level of the colonic mucosa. Labeling was carried out with the antibody directed against the extracellular domain of the receptor. By immunohistochemistry, we demonstrated that the anti- $\mu$ -opioid receptor antibody stained the basolateral membrane of epithelial cells in the upper half of the crypt (Fig. 1A). With anti-MOR-1 antibody (Santa Cruz Biotechnology) and with H-300 anti-Muc2 antibody, we performed double immunostaining.  $\mu$ -Opioid receptors were evidenced in some goblet cells in the upper half of the crypt (Fig. 1A).

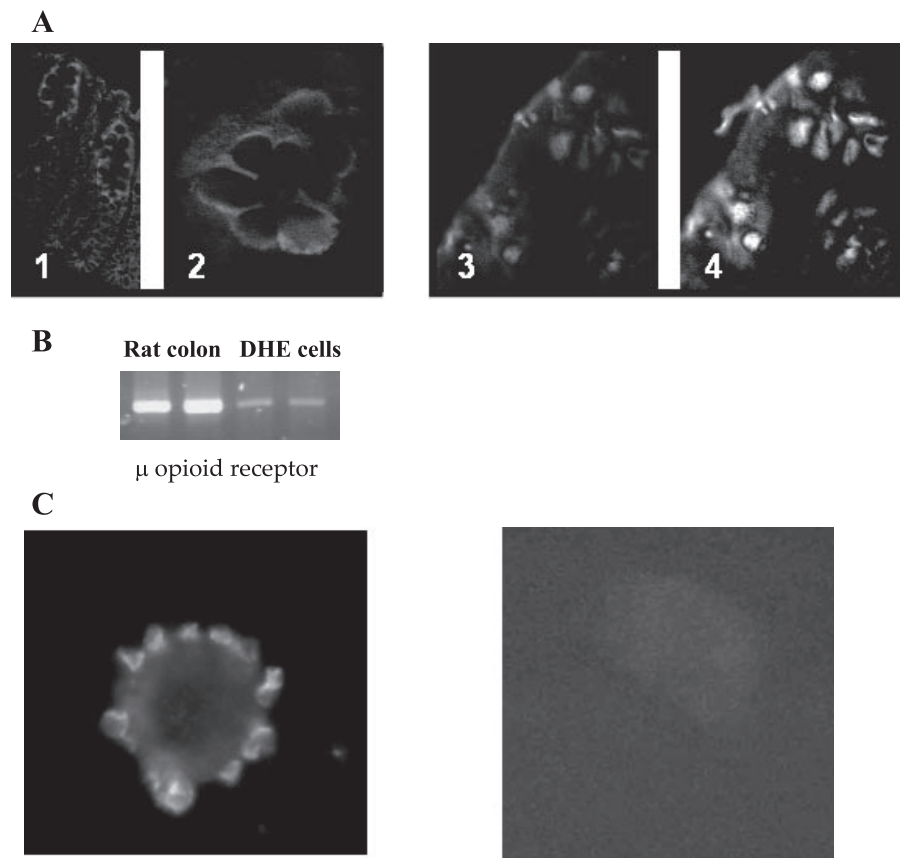
To assess the direct effect  $\beta$ -CM-7 on goblet cells, we used rat intestinal DHE cells, which synthesize and secrete mucins. By RT-PCR, we evidenced the presence of the transcripts of  $\mu$ -opioid receptors in DHE cells. On the basis of the primers used, the size of the expected amplicons was 569 bp (Fig. 1B), and, after cDNA sequencing, these products were found to be identical to the  $\mu$ -opioid receptor cDNA sequence. By immunohistochemistry, we also demonstrated the expression of  $\mu$ -opioid receptors on the DHE cell surface. The staining pattern was located predominantly in clusters on the membranes of DHE cells (Fig. 1C).

#### *$\beta$ -CM-7 Stimulates the Secretion of Mucin in DHE Cells*

To demonstrate a possible direct effect of  $\beta$ -CM-7 on mucin secretion, we exposed DHE cultures to  $\beta$ -CM-7 ( $10^{-4}$  M) for 30 min to 24 h. As shown in Fig. 2, the overall release of mucinlike glycoprotein under the influence of  $\beta$ -CM-7 was not modified after 30 min of stimulation but raised after 2 h of exposure to  $\beta$ -CM-7. This effect was maximum after 8 h of stimulation ( $227 \pm 12\%$  of controls,  $P < 0.05$ ). Using an ELISA for rMuc2, we found that  $10^{-4}$  M  $\beta$ -CM-7 induced a rise in rMuc2 secretion after 8 h of treatment ( $192 \pm 4\%$  of controls;  $P < 0.05$ ).



Fig. 1.  $\mu$ -Opioid receptors (MOR) are expressed in rat colonic epithelium and intestinal DHE cells. **A:** 4- $\mu$ m-thick histological sections of rat colonic mucosa. 1, the tissue section was incubated with the anti-MOR antibody followed by an anti-rabbit rhodamine-conjugated secondary antibody. The primary antibody, prepared by J. Garzon, was directed against the extracellular domains of receptors. The immunohistochemical localization of MOR was shown by deposit of fluorescence in the upper half of the crypt. 2, en face preparation of rat colonic mucosa. The immunostaining with the same primary antibody showed the characteristic shape of goblet cells. 3 and 4, double immunostaining of MOR (3) and rMuc2 (4) on rat colonic mucosa. The colonic section was incubated with anti-MOR-1 antibody (Santa Cruz Biotechnology), followed by rhodamine-conjugated secondary antibody. The section was then incubated with H-300 anti-Muc2 antibody, followed by FITC-conjugated secondary antibody. The green fluorescent reaction highlighted the presence of rat mucin (rMuc)2 in goblet cells, whereas the red fluorescent reaction showed the presence of MOR in some goblet cells but not in all. **B:** evidence for MOR mRNA in DHE cells. Electrophoretic PCR gels demonstrating the presence of MOR transcripts in DHE cells. Transcript expression in the rat colon is shown as reference. **C:** immunocytochemistry of MOR using rhodamine immunofluorescence. *Left*, evidence of clustered MOR-like immunoreactivity at the surface of DHE cells. *Right*, negative control with the anti-MOR antibody omitted.



#### $\beta$ -CM-7 Stimulates the Expression of rMuc2 and rMuc3 in DHE Cells

To determine whether  $\beta$ -CM-7 could raise mucin gene expression, we treated DHE cells with  $\beta$ -CM-7 at 37°C for 24 h, after which time total RNA was isolated and mucin mRNA levels were analyzed by RT-PCR. Addition of  $\beta$ -CM-7 ( $10^{-4}$  M) to the incubation medium for 24 h induced an increase in rMuc2 and rMuc3 mRNA levels (Fig. 3). In contrast,  $\beta$ -CM-7 did not modify the expression of rMuc1,

rMuc4, and rMuc5AC. RT-PCR products of cyclophilin A mRNA, used as internal control, were unaffected by  $\beta$ -CM-7.

The dose-response effect of  $\beta$ -CM-7 ( $10^{-6}$  to  $10^{-4}$  M) on mRNA levels of rMuc2 and rMuc3 was further determined

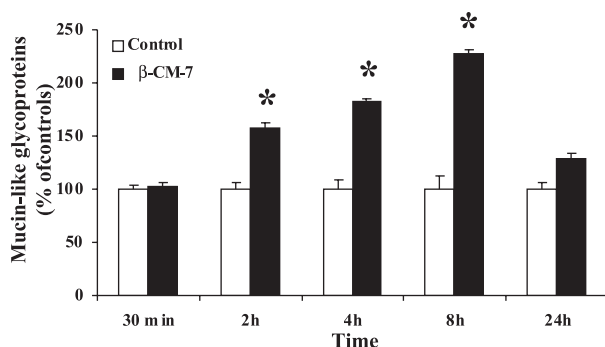


Fig. 2. Time-course effect of  $\beta$ -casomorphin-7 ( $\beta$ -CM-7) on the secretion of mucinlike glycoprotein in DHE cells. After 24 h of serum starvation, the cells were incubated without (control) or with  $\beta$ -CM-7 for 30 min to 24 h. The amount of mucinlike glycoprotein in culture media was measured by enzyme-linked lectin assay (ELLA) using biotinylated wheat germ agglutinin. The data are expressed as mucinlike glycoprotein secretion as a percentage of control. Each point represents the mean  $\pm$  SE of 3 experiments performed in triplicate. \* $P < 0.05$  vs. control.

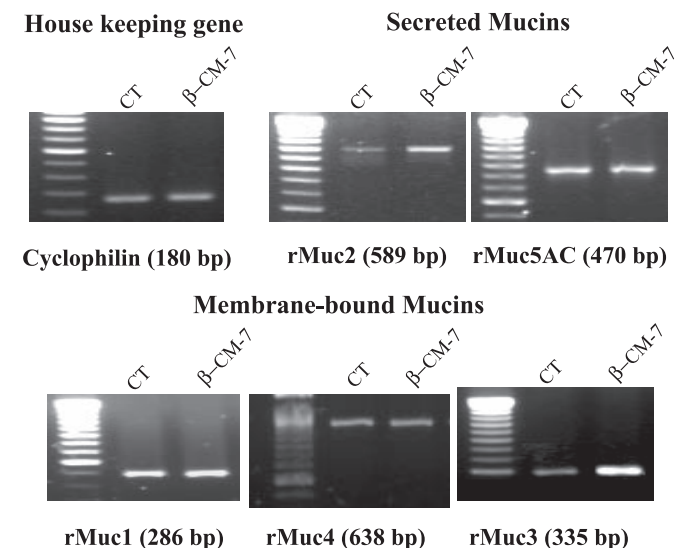


Fig. 3.  $\beta$ -CM-7 ( $10^{-4}$  M) specifically modulates rMuc2 and rMuc3 mRNA expression in the rat intestinal DHE cell line. After overnight serum starvation, the cells were incubated without (CT) or with  $\beta$ -CM-7 at 37°C for 24 h. Amplified PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. Images are representative of 3 separate experiments performed in triplicate. Cyclophilin A mRNA was used as reference.

after 24 h of treatment. A representative set of original experimental results is shown in Fig. 4A. RT-PCR analysis using Scion image showed that β-CM-7 induced a dose-dependant increase in rMuc2 and rMuc3 mRNA levels compared with controls (Fig. 4B). The response obtained with  $10^{-4}$  M β-CM-7 was at  $183 \pm 14$  and  $172 \pm 8\%$  of controls for rMuc2 and rMuc3, respectively.

A time-course response was then performed on the effect of β-CM-7 ( $10^{-4}$  M) on the expression of rMuc2 and rMuc3. β-CM-7 increased the level of rMuc2 and rMuc3 mRNA after 2 ( $225 \pm 16\%$  of controls) and 4 h ( $208 \pm 8\%$  of controls) of stimulation, respectively. This effect was maintained after 24 h of treatment (Fig. 5). To precisely quantify the effect of β-CM-7 on the expression of rMuc2 in DHE cells, we performed quantitative RT-PCR. The level of rMuc2 mRNA was twofold increased following 8 h of treatment with β-CM-7 ( $10^{-4}$  M;  $P < 0.05$ ; Fig. 5).

#### Mechanisms Involved in β-CM-7-Induced Mucin Expression in DHE Cells

A μ-opioid agonist increases the expression of mucin genes. To determine whether the μ-opioid receptor was involved in mucin expression and secretion, DHE cells were treated for 8 h

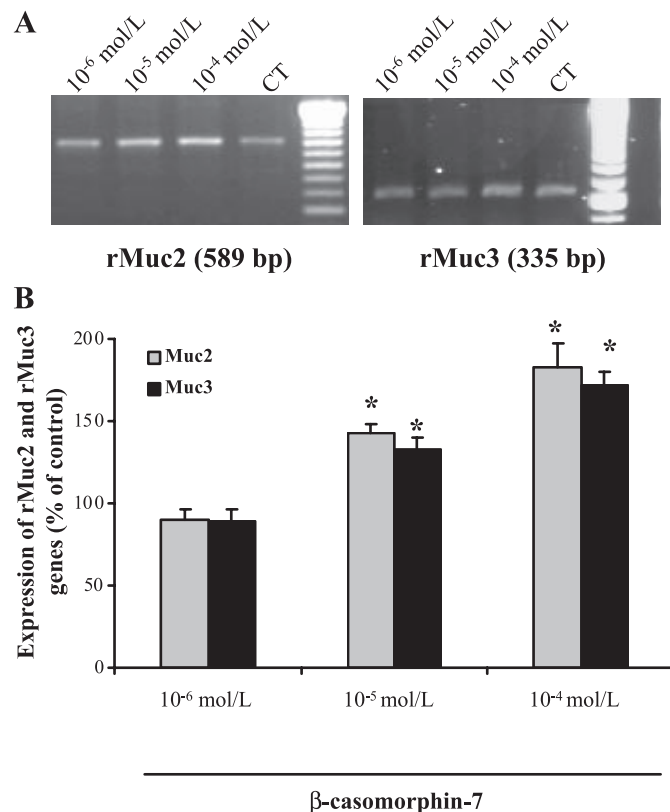


Fig. 4. β-CM-7 dose dependently increases the level of rMuc2 and rMuc3 mRNA in DHE cells. A: DHE cells were exposed to β-CM-7 for 24 h. Total RNA was isolated, and mucin mRNA levels were analyzed by RT-PCR. Amplified PCR fragments were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. Representative gels of 3 experiments performed in triplicate are shown. B: rMuc2 and rMuc3 mRNA levels (expressed as a percentage of associated controls). The gels obtained from semiquantitative RT-PCR were pixelized and densitometrically analyzed with Scion image software. Each point represents the mean  $\pm$  SE of 3 experiments performed in triplicate. \* $P < 0.05$  vs. control.

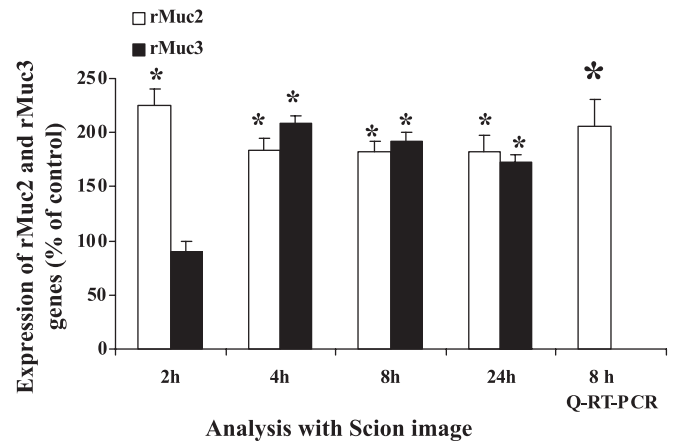


Fig. 5. Time-related effect of  $10^{-4}$  M β-CM-7 on the level of rMuc2 and rMuc3 mRNA in DHE cells. Cells were exposed to β-CM-7 in the medium for 2–24 h. The gels obtained from semiquantitative RT-PCR were pixelized and densitometrically analyzed. rMuc2 from the 8 h-stimulated cells was analyzed by quantitative RT-PCR (Q-RT-PCR) with cyclophilin A as an internal control. The expression of rMuc2 was normalized to cyclophilin A mRNA level in each sample. The results are expressed as a percentage of associated controls (means  $\pm$  SE). \* $P < 0.05$  vs. control.

with DAMGO, a μ-specific agonist. As shown in Fig. 6,  $10^{-6}$  M DAMGO induced an increase in rMuc2 mRNA levels. Likewise, the overall release of mucinlike glycoprotein under the influence of DAMGO was significantly increased ( $173 \pm 20\%$  of controls,  $P < 0.05$ ) and was quite similar to that after β-CM-7 stimulation.

Cyprodime inhibits the increase in the expression of rMuc2 and rMuc3 induced by β-CM-7. We then studied the effect of a μ-opioid antagonist (cyprodime;  $10^{-5}$  M) on the rise in rMuc2 and rMuc3 mRNA levels induced by β-CM-7 in DHE cells. The cells were pretreated for 30 min at 37°C with cyprodime before the addition of β-CM-7 ( $10^{-4}$  M, 8 h). Data obtained with quantitative RT-PCR showed that the effect of β-CM-7 on rMuc2 expression was inhibited by cyprodime, whereas the antagonist alone had no effect on the rMuc2

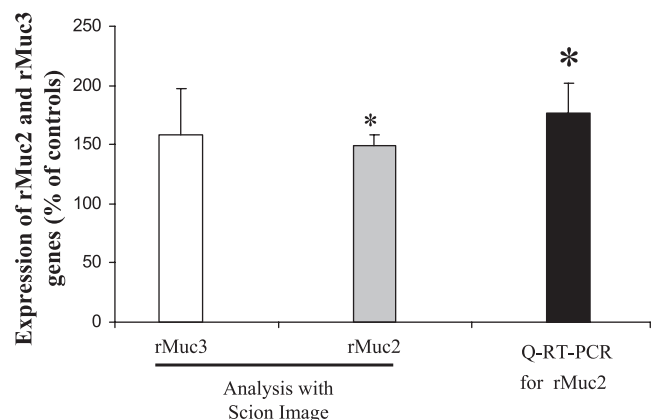


Fig. 6. Rat mucin-secreting DHE cells are responsive to an μ-opioid agonist. The effect of (D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,glycylol<sup>5</sup>)enkephalin (DAMGO;  $10^{-6}$  M) on rMuc2 and rMuc3 after 8 h of stimulation was studied with semiquantitative RT-PCR and the effect on rMuc2 was quantified by Q-RT-PCR. The expression of rMuc2 was normalized to cyclophilin A mRNA level in each sample. The results are presented as the percent increase of the untreated control (means  $\pm$  SE). All results are representative of 3 separate experiments performed in triplicate. \* $P < 0.05$  vs. control.

mRNA level (Fig. 7A). Cyprodime also inhibited the rise in the rMuc3 mRNA level induced by  $\beta$ -CM-7 (analysis with Scion image, data not shown). As shown in Fig. 7B, cyprodime blocked  $\beta$ -CM-7-induced mucinlike glycoprotein secretion. Comparable cyprodime inhibitions were obtained after stimulation with DAMGO (data not shown).

#### $\beta$ -CM-7 Stimulates MUC5AC Expression and Mucin Secretion in HT29-MTX Cells

To establish whether  $\beta$ -CM-7 can also modulate mucins in humans, we extended our study to HT29-MTX cells, a human colonic cell line known to synthesize and secrete mucins. We first found that, as with DHE cells, HT29-MTX cells exhibited immunopositivity for  $\mu$ -opioid receptors on their cell membranes (Fig. 8A). By RT-PCR, the transcripts of  $\mu$ -receptors were also evidenced (data not shown). The major mucin produced by HT29-MTX cells is MUC5AC. As shown in Fig. 8B, time-course experiments showed that the addition of  $\beta$ -CM-7 ( $10^{-4}$  M) into the incubation medium of HT29-MTX cells elicited an increase in the level of MUC5AC mRNA. By quantitative RT-PCR, we determined that the maximal response was at  $176 \pm 14\%$  ( $P < 0.05$ ) after 24 h of treatment.

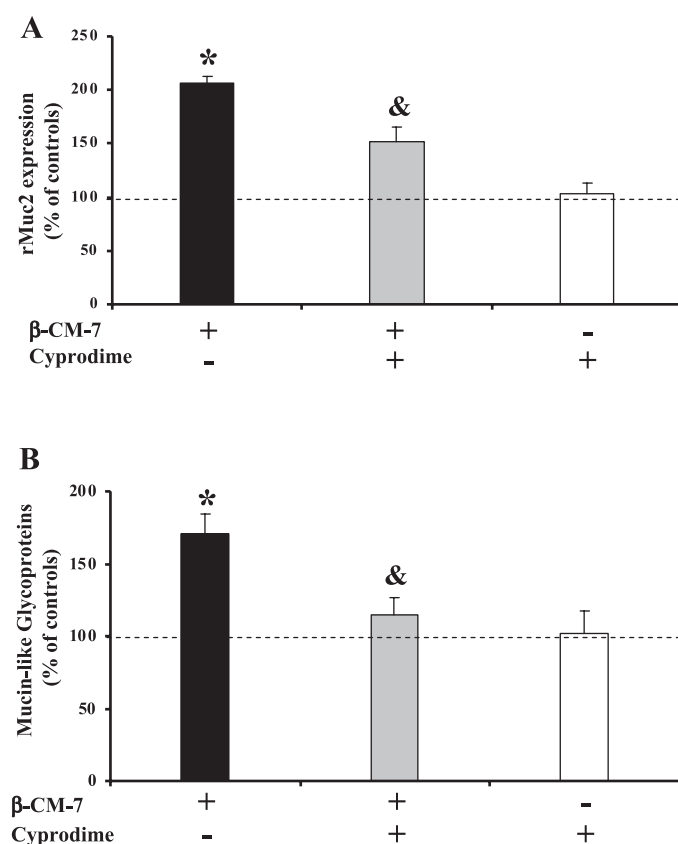


Fig. 7. Rat mucus-secreting DHE cells are responsive to  $\beta$ -CM-7 through a  $\mu$ -opioid-dependent pathway. DHE cells were incubated for 30 min with the  $\mu$ -opioid antagonist cyprodime ( $10^{-5}$  M) either alone or in association with  $\beta$ -CM-7 ( $10^{-4}$  M) for 8 h. A: rMuc2 mRNA levels in DHE cells. rMuc2 was analyzed by Q-RT-PCR with cyclophilin A as an internal control. The expression of rMuc2 was normalized to cyclophilin A mRNA level in each sample. The results are presented as the percent increase with respect to the untreated control (means  $\pm$  SE). B: mucinlike glycoprotein secretion was determined by ELLA. Each point represents the mean  $\pm$  SE of 3 experiments performed in triplicate. \* $P < 0.05$  vs. control; & $P < 0.05$  vs. agonist alone.

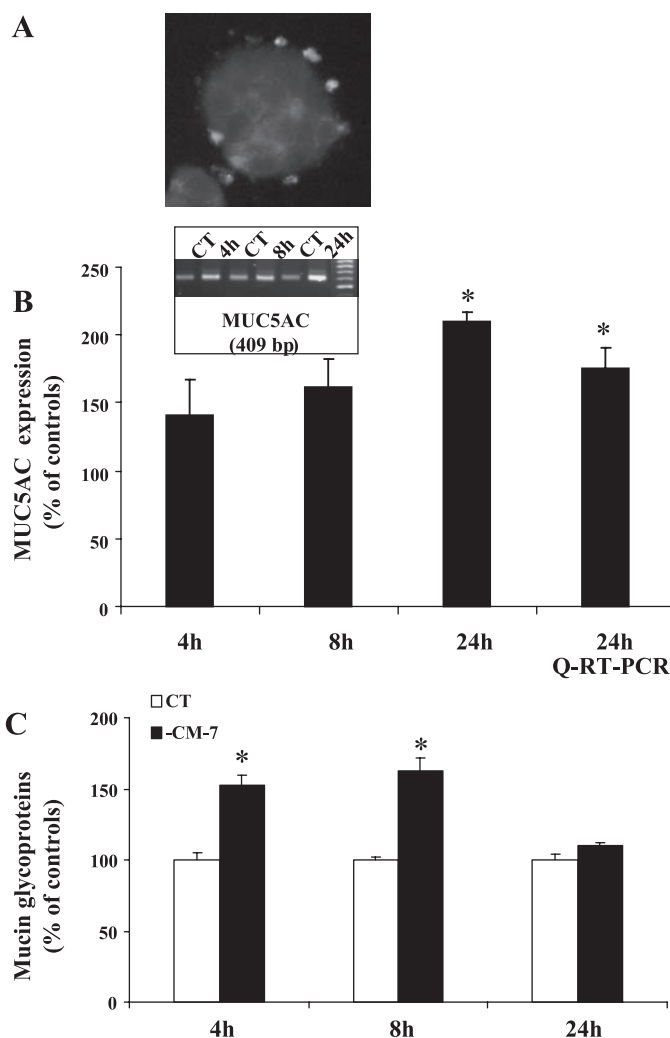


Fig. 8. HT29-MTX cells are responsive to  $\beta$ -CM-7 ( $10^{-4}$  M). A: immunocytochemistry of the MOR using rhodamine immunofluorescence. B: effect of  $\beta$ -CM-7 ( $10^{-4}$  M) on mucin (MUC)5AC mRNA level after 4–24 h of exposure. Total RNA was extracted and then submitted to semiquantitative RT-PCR. Gels were analyzed by Scion image software. MUC5AC was also determined by Q-RT-PCR after a 24-h exposure. All results are representative of 3 separate experiments performed in triplicate. Inset, PCR products after electrophoresis in a 2% agarose gel. C: overall release of mucinlike glycoprotein analyzed by ELLA. All results are representative of 3 separate experiments performed in triplicate. \* $P < 0.05$  vs. control.

$\beta$ -CM-7 ( $10^{-4}$  M) also induced mucinlike glycoprotein secretion (Fig. 8C). The maximal response was obtained after 8 h of stimulation with  $\beta$ -CM-7 ( $163 \pm 9\%$  of controls;  $P < 0.05$ ). Using an ELISA for MUC5AC, we detected a similar rise in the secretion of this mucin ( $169 \pm 3\%$  of controls;  $P < 0.05$ ).

#### DISCUSSION

The present study provides original data about the in vitro effect of a milk bioactive peptide,  $\beta$ -CM-7, on mucin expression and secretion in rat and human colon gobletlike cells. This  $\beta$ -CM-7 stimulation is dependent on  $\mu$ -opioid receptor activation. As far as we are aware, this is the first report that an alimentary peptide, as well as an opioid agonist, directly activates intestinal mucin-secreting gobletlike cells. It is of

note that luminal concentrations of β-CM-7 after an ingestion of milk have not been determined, but the dose of β-CM-7 used in our study ( $10^{-4}$  M) was equivalent to what could be theoretically obtained from casein hydrolysates at a concentration of 0.5% in the intestinal lumen.

β-CMs are a family of opioid peptides derived from bovine β-casein. These peptides, which are encrypted within the sequence of the parent protein, are released by enzymatic proteolysis during gastrointestinal digestion or during food processing (33–35). Identical sequences exist in ovine β-casein, and peptides with similar activity are derived from human β-casein (33–35). Interestingly, β-CMs, which have been detected in the small intestine of adult humans and in the plasma of newborn calves after the ingestion of bovine milk (49), are resistant to the actions of gastrointestinal enzymes due to a high content of proline residues. They could thus elicit physiological effects and may represent natural agonists for opioid receptors. In the present study, we demonstrated the presence of μ-opioid receptors on the basolateral membrane of goblet cells in the upper half of the colonic crypt, where cells achieve differentiation, as well as in cell lines. These receptors were clustered, which suggests proper targeting and anchoring to the cytoplasmic membrane, a prerequisite for the receptor to be functional (22). Consistent with this assumption, β-CM-7 increased the levels of rMuc2 and rMuc3 mRNA in DHE cells as well as the overall discharge of mucin after 2 h of treatment. These findings were reproduced by the μ-agonist DAMGO. Finally, pretreatment of the cells with a specific μ-opioid antagonist, cyprodime, inhibited the effect of β-CM-7 or DAMGO on the expression of rMuc2 and on the secretion of mucin, confirming the involvement of μ-opioid receptors in the response of DHE. Consequently, it is conceivable that opioid peptides released by the digestion of milk protein in the intestinal lumen could act locally on mucus cells to protect the colonic mucosa.

The prominent localization of opioid receptors in the gut is the myenteric and submucosal plexus (1). It is interesting to note, however, that, in keeping with our results, the presence of μ-opioid receptors has also been demonstrated on epithelial cells of the rat, pig, and guinea pig (26, 30, 36, 38), suggesting that opioid agonists may act directly on the intestinal epithelium to regulate its functions. Supporting this hypothesis, it was previously demonstrated that opioid peptides can regulate hydroelectrolytic secretion by both acting on enteric neurons and a direct effect on epithelial cells (21, 26, 36). Similarly, we recently showed in a rat model of the isolated vascularly perfused jejunum that β-CM-7 induced a strong and fast (in the first 30 min of stimulation) secretion of intestinal mucus through activation of the enteric nervous system and opioid receptors (10, 46). β-CMs could thus regulate the function of intestinal goblet cells via two distinct but complementary modes of action: by a direct pathway to increase the expression of the intestinal mucin genes and by an indirect nervous pathway to induce a rapid increase of the secretory activity of intestinal goblet cells. The complementary effects of opioid peptides on goblet cells might be an important facet of intestinal defense. Thus opioid peptides induce a strong and rapid secretion of mucus, but they also help to reconstitute the intracellular store of goblet cells by increasing mucin gene expression and maintaining the potential of intestinal defense. It may be assumed that the elaborate way of control of goblet

cells we observed with β-CM-7 also triggers the effects of other opioid peptides from milk (other casomorphins, lactorphins, etc.) but also from endogenous origin.

What is the physiological meaning of the localization of opioid receptor on mucin-producing cells? In the gastrointestinal tract, opioid peptides (β-endorphin, enkephalins, and dynorphin) are primarily expressed in neurons of the myenteric and submucous plexus, and some opioid-immunoreactive fibers have been observed through the different areas of the mucosa, especially around crypts (1, 16). Endogenous opioid peptides also appear to be present in immune cells (1, 44) and in enteroendocrine cells, including enterochromaffin cells of the intestine and gastrin cells of the antrum (6, 37, 42). Accordingly, the regulation of mucins we observed here with opioid agonists should also be relevant in situations of physiological activation of the enteric nervous system as well as of enteroendocrine cells or immune cells.

In DHE cells, β-CM-7 and DAMGO increased the level of transcripts of rMuc2 and rMuc3 but did not alter rMuc1, rMuc4, and rMuc5AC mRNAs. rMuc2 is the main mucin secreted by goblet cells in the gut and is expressed at a high level in the ileum and colon (32). With rMuc1 and rMuc4, rMuc3 is one of the prominent membrane-associated mucins expressed in the rat intestine and colon (14, 45). β-CM-7 thus increased the mRNA level of two components of the mucosal protection: a membrane-associated mucin and a secreted mucin. In our study, rMuc2 mRNA was readily increased on β-CM-7 stimulation, whereas rMuc3 was increased after only 4 h, thus suggesting the involvement of specific mechanisms of activation. A faster rMuc2 than rMuc3 gene activation is not surprising considering that goblet cells have to prevent the depletion of their mucus stores, whereas membrane-associated mucins provide a more static protection of the intestinal mucosa.

Interestingly, we could extend our data obtained in a rat cell line to human gobletlike HT29-MTX cells (28). Indeed, β-CM-7 induced an increase of MUC5AC expression, the major secreted mucin of this cell line, as well as secretion of mucin in the medium, thus suggesting that these responses may take place in several species. These findings may have health implications. Because milk constitutes the only source of protein for neonates, the biological consequences of the increase in mucin expression by β-CMs may be to reinforce or to facilitate the development of a protective mucus gel.

In conclusion, this study demonstrates that an opioid peptide from milk, β-CM-7, induces the secretion of mucin as well as the expression of rMuc2 and rMuc3 in DHE rat cells and the expression of MUC5AC in HT29-MTX human colonic gobletlike cells. These effects in DHE cells were reproduced by the μ-opioid agonist DAMGO and were mediated through a μ-opioid pathway. These results suggest that, in vivo, β-CM-7 can modulate intestinal mucins through a direct effect on goblet cells. Milk opioid-derived peptides could thus provide new dietary prospects for improving gastrointestinal protection in the neonate but also in the adult. Our data also support the growing evidence that the μ-opioid pathway is important in intestinal defense.

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