Opioid peptides encrypted in intact milk protein sequences

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Opioid agonistic and antagonistic peptides which are inactive within the sequence of the precursor milk proteins can be released and thus activated by enzymatic proteolysis, for example during gastrointestinal digestion or during food processing. Activated opioid peptides are potential modulators of various regulatory processes in the body. Opioid peptides can interact with subepithelial opioid receptors or specific luminal binding sites in the intestinal tract. Furthermore, they may be absorbed and then reach endogenous opioid receptors.

Opioid peptides: Milk protein

Introduction

Milk proteins are potential sources of opioid agonistic and antagonistic peptides. The structures of biologically active sequences were obtained from *in vitro* enzymatic and/or by *in vivo* gastrointestinal digests of the appropriate precursor proteins; chemical synthesis has been carried out to confirm the sequence of potential bioactive peptides (for reviews, see Teschemacher *et al.* 1990, 1994, 1997; Meisel, 1997*a*).

General structural features

Opioid peptides, i.e. opioid receptor ligands with agonistic activity, originate from different milk proteins and exert naloxone-inhibitable opioid activities in both receptor studies and during bioassays (Brantl *et al.* 1981). Milk-protein derived opioid peptides have been designated as 'atypical' opioid peptides (Teschemacher *et al.* 1994). These opioid peptides have N-terminal sequences different from that of the 'typical' endogenous opioid peptides, e.g. enkephalins, endorphins and dynorphins. 'Typical' opioid peptides are derived from three precursor molecules, proenkephalin, prodynorphin and proopiomelanocortin (Höllt, 1983). The common structural feature among endogenous and exogenous opioid peptides is the presence of a tyrosine residue at the amino terminal end (except α-casein opioids) and the presence of another aromatic residue, e.g. phenylalanine or tyrosine, in the third or fourth position (Table 1). This is an important structural motif which fits into the binding site of opioid receptors. The negative potential, localized in the vicinity of the phenolic hydroxyl group of tyrosine, seems to be essential for opioid activity. Removal of the tyrosine residue results in a total absence of bioactivity (Chang *et al.* 1981). The proline residue in the second position (Pro^2) is crucial for the biological activity of opioid peptides because it is reported to maintain the proper orientation of the Tyr and Phe side chains (Mierke *et al.* 1990).

Opioid antagonists are peptides related to 'atypical' opioid peptides, i.e. they are opioid receptor ligands sharing several characteristics with agonistic opioid peptides derived from milk proteins (Teschemacher *et al.* 1994). They are not found in the sequence of endogenous precursors and their N-terminal amino acid sequence is not identical with that of the 'typical' opioid peptides.

Occurrence in the sequence of milk proteins

The major exogenous opioid peptides, i.e. β-casomorphins, are fragments of the β-casein sequence 60–70 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu) (Table 1) and have been characterized as μ-type ligands (Teschemacher *et al.* 1994, 1997). β-Casomorphins were found in analogous positions in sheep, water buffalo and human β-casein (for review, see Fiat & Jollès, 1989; Teschemacher *et al.* 1990; Meisel & Schlimme, 1996; Meisel, 1997*a*,*b*). An analogue isolated from a commercial hydrolysate of bovine casein, β-casomorphin-4-amide, designated as morphiceptin, is a highly potent μ-selective opioid peptide (Chang *et al.* 1981). Three α-casein-derived exorphins corresponding to bovine α_{S1}-casein peptide fragments 90–96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu), 90–95 and 91–96 are δ-selective receptor ligands (Loukas *et al.* 1983, 1990; Pihlanto-Leppälä *et al.* 1994). Whey protein-derived peptides which behave as μ-opioid receptor agonists with low potency are the α-lactorphin (Tyr-Gly-Leu-Phe) and β-lactorphin (Tyr-Leu-Leu-Phe) corresponding to residues 50–53 in both bovine and human α-lactalbumin and 102–105 in bovine β-lactoglobulin, respectively (Chiba & Yoshikawa, 1986; Antila *et al.* 1991). Another whey protein-derived opioid

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Table 1. Examples of opioid peptides derived from bovine milk proteins (adapted from Meisel & Bockelmann, 1999 and Antila *et al.* 1991)

Bioactive peptide	Sequence*	Precursor protein (fragment)	IC_{50}† (μmol/l)	Theoretical yield of bioactive peptide obtainable from 1 g precursor protein (mg)
Opioid agonists				
β-Casomorphin-7	YPFPGPI	β-Casein (f60–66)	14.0	33.0
β-Casomorphin-5	YPFPG	β-Casein (f60–64)	1.1	24.2
α_{s1}-Casein-exorphin	RYLGYLE	α_{s1}-Casein (f90–96)	1.2	38.7
α-Lactorphin	YGLF	α-Lactalbumin (f50–53)	67.0	35.2
β-Lactorphin	YLLF	β-Lactoglobulin (f102–105)	38.0	30.2
Serorphin	YGFNA	Serum albumin (f399–404)	85.0	10.5
Opioid antagonists				
Casoxin A	YPSYGLNY	κ-Casein (f35–42)	400.0	51.4
Casoxin B	YPYY	κ-Casein (f58–61)		31.8
Casoxin C	YIPIQYVLSR	κ-Casein (f25–34)	50.0	65.8

* The one-letter amino acid code is used.
† Peptide concentration required to inhibit ^{3}H-ligand binding by 50 %.

peptide, serorphin (Tyr-Gly-Phe-Asn-Ala), was isolated from fragment 399–404 of serum albumin (Tani *et al.* 1994).

Opioid antagonists have been found in bovine and human κ-casein (casoxins) and in α_{S1}-casein (Chiba *et al.* 1989; Yoshikawa *et al.* 1994). Various synthetic casoxins were isolated as C-terminally methoxylated peptides, e.g. the derivatives corresponding to κ-casein sequences 33–38 (Ser-Arg-Tyr-Pro-Ser-Tyr OCH_3), 34–38 and 35–38 (Chiba & Yoshikawa, 1986). The chemically modified casoxins were more active than the non-methoxylated fragments. The tryptic fragment corresponding to residues 25–34 (Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg) of bovine κ-casein, known as casoxin C, showed a relatively high opioid antagonistic activity in comparison to the esterified peptides (Chiba *et al.* 1989). Casoxins are opioid receptor ligands of the μ-type with relatively low antagonistic potency as compared with naloxone. They also bind to κ-receptors where extension of the N-terminal amino acid sequence beyond the tyrosine residue seems to influence binding to κ-type receptors. Opioid antagonists were also found in human lactoferrin (Yoshikawa *et al.* 1988). These peptides, named lactoferricin A (fragment 339–344), B (fragment 544–548) and C (fragment 681–687), behaved quite similarly to casoxins, i.e. C-terminally methoxylated μ-opioid receptor-selective antagonists with moderate potency (Teschemacher *et al.* 1997).

Functional significance

Mammalian opioidergic systems consist of opioid receptors and their endogenous ligands, the opioid peptides. Depending on their location, their physiological significance appears to be related to a considerable number of neuroendocrine regulatory functions. Opioid receptors (μ-, δ- and κ-type) are located in the nervous, endocrine and immune systems as well as in the intestinal tract of the mammalian organism and can interact with their endogenous ligands as well as with exogenous opioids and opioid antagonists (Teschemacher *et al.* 1994). Thus, opioidergic systems are open to all kinds of interferences from exogeneous opioids or opioid antagonists (Teschemacher *et al.* 1994). Modulation of social behaviour (Panksepp *et al.* 1984; Paroli, 1988) and analgesic effects (Chang *et al.* 1982; Matthies *et al.* 1984) were observed following intracerebral administration of opioid peptides with agonistic activity, e.g. β-casomorphins, to experimental animals. Orally administered milk protein-derived opioid peptides have been demonstrated to influence postprandial metabolism by stimulating secretion of insulin and somatostatin (Schusdziarra *et al.* 1983*a*,*b*), to modulate intestinal transport of amino acids (Brandsch *et al.* 1994), to prolong gastrointestinal transit time and to exert antidiarrhoeal action (Daniel *et al.* 1990*a*,*b*). Evidence has accumulated that the enhancement of net water and electrolyte absorption by β-casomorphins in the small and large intestine is a major component of their antidiarrhoeal action, which could be mediated via subepithelial opioid receptors or through specific luminal binding sites at the brush border membrane (Tomé *et al.* 1987; Brandsch *et al.* 1994). β-Casomorphins may also affect the human mucosal immune system, possibly via opiate receptors in lamina propria lymphocytes (Elitsur & Luk, 1991).

The presence of opioid peptides in a living system or, at least, of the precursors from which these peptides could be liberated, is a prerequisite for any functional role in the system. It can be shown that relatively high amounts of bioactive peptides could potentially be produced during the ingestion of 1 g of each of the major casein and whey protein components (Table 1). Many opioid peptides are biologically very potent. Therefore, even nutritionally insignificant amounts of liberated peptides might be sufficient to exert physiological effects. These peptides may enter peripheral blood intact and exert systemic effects or they may produce local effects in the gastrointestinal tract. Results indicating the liberation of β-casomorphins from β-casein under *in vivo* conditions have been obtained from several studies. Evidence for the liberation of β-casomorphins from β-casein into the gastrointestinal lumen of mammals after milk intake has also been obtained. Peptides were found in the small intestinal contents of adult humans following cows' milk intake, which were identified by radioimmunological and chromatographical methods as β-casomorphins (Svedberg *et al.* 1985). Moreover, β-casomorphin-11 has been identified and chemically characterized in the duodenal chyme of Göttingen minipigs after feeding with bovine casein (Meisel, 1986). β-Casomorphins are claimed to be rapidly degraded once

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they enter the bloodstream. However, the presence of β-casomorphin-7 immunoreactive material has been demonstrated in the plasma of newborn calves following their first milk intake (Umbach *et al.* 1985). This material revealed a similar molar mass as β-casomorphin-11 and thus has been considered as a β-casomorphin precursor. Such pre-casomorphins could reach any potential site of action in the system to elicit physiological effects following liberation of the protected active sequence from the precursor molecule. Opioid casein fragments have not been detected in the plasma of adult mammals (Umbach *et al.* 1985; Teschemacher *et al.* 1986). Thus, only the neonatal intestine appears to be permeable to (pre-)casomorphins; in adult systems, the intestinal brush border membrane seems to be the main target site for the physiological effects of food-derived opioid peptides.

In addition to the possible liberation of bioactive peptides during intestinal proteolysis, such peptides may already be generated during manufacture of several milk products and thus be ingested as food components. For example, partially hydrolysed milk proteins for hypoallergenic infant formulas and for clinical applications in enteral nutrition consist exclusively of peptides. Furthermore, it has been demonstrated that a number of caseolytic bacterial species used in the production of some types of cheese and other milk products can produce casomorphins (Hamel *et al.* 1985). Several opioid peptides derived from α_{S1}-, β-casein and α-lactalbumin were released by pepsin/trypsin hydrolysis of *Lactobacillus GG* fermented UHT milk (Rokka *et al.* 1997). It has been found that the extracellular P_I-type proteinase of *Lactococcus lactis* hydrolyses more than 40 % of the peptide bonds of β-casein resulting in the formation of more than 100 different oligopeptides including a fragment of the β-casein sequence 60–68 which is part of β-casomorphin-11 (Juillard *et al.* 1995). Data obtained from 'non-starter' lactic acid bacteria (*Lactobacillus* ssp.) present in fermented milk products and in the human intestine indicate that their proteolytic system is comparable to *Lactococcus lactis*. No data are available on the action of the proteolytic enzymes from lactic acid bacteria in the human gut after ingestion of fermented milk products.

Irrespective of the potential function of milk-derived opioids as exogenous regulators, it is also possible that certain peptides from milk may directly influence the mother. For example, casomorphins can be liberated in the mammary gland, transferred to the blood and then reach endogenous opioid receptors (Teschemacher & Koch, 1990). In this way, casomorphins may participate in the endocrinic regulation of pregnancy, e.g. by stimulation of prolactin release (Yen *et al.* 1985). The cardiovascular system in pregnant or lactating mammals may also be a target for casomorphin action. These peptides can exert a positive inotropic and antiarrhythmic effect and thus may have a cardioprotective function (Mentz *et al.* 1990; Teschemacher & Koch, 1990). The physiological significance of these effects is as yet not clear.

Dietary and pharmaceutical applications

β-Casomorphins have been produced by genetic engineering techniques followed by enzymatic or chemical cleavage of the microbial fusion protein to liberate the required peptide (Carnie *et al.* 1989). These recombinant β-casomorphins are intended for oral administration in order to increase animal performance, e.g. weight gain or milk yield. As yet, no meaningful application in human nutrition has been described.

Several attempts were made to synthesize modified β-casomorphin sequences in order to find pharmacologically active peptides with higher analgesic potency, altered side-effects and longer duration of action. A considerable increase in analgesic or antidiarrhoeal activity in dogs was obtained by substitution of L- with D-amino acids, e.g. Pro^2 and Pro^4, and by C-terminal amidation (Matthies *et al.* 1984; Daniel *et al.* 1990*b*; Mansfeld *et al.* 1990; Erll *et al.* 1994). Examples of chemically modified potent opioid peptides include morphiceptin (β-casomorphin-4-amide) and casokefamide (D-$Ala^{2,4}$,Tyr^5-β-casomorphin-5-amide). Modifications to the natural casomorphins do not only influence the affinity of the resulting analogues for opioid receptors, but also alter their pharmacokinetics, particularly their inactivation by proteolytic/peptideolytic enzymes: substituted β-casomorphins have been shown to be more resistant to enzymatic attack and exhibit higher opioid potency than the natural peptides (Tomé *et al.* 1987; Daniel *et al.* 1990*b*).

Conclusions

The bioactivities of opioid peptides encrypted in major milk proteins are latent until released and activated by enzymatic proteolysis, e.g. during gastrointestinal digestion or food processing. It is evident from many studies that opioid peptide fragments originating from milk proteins are potential regulatory compounds and modulators of various regulatory processes in the body. Nevertheless, more research is needed to fully understand the physiological significance of milk-protein derived opioid peptides. β-Casomorphins and chemically modified analogues have already been considered for interesting applications as supplements for animal feed and as pharmaceutical preparations.

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